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Award Number: W81XWH-06-C-0360

TITLE: Discovery of Novel Virulence Factors of Biothreat Agents: Validation of the Phosphoproteome-Based Approach

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REPORT DATE: June 2008

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
30-06-2008	Annual	1 JUN 2007 - 31 MAY 2008

4. TITLE AND SUBTITLE

Discovery of Novel Virulence Factors of Biothreat Agents: Validation of the Phosphoproteome-Based Approach

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-06-C-0360

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

George Mason University
Fairfax, VA 22030

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

See next page.

15. SUBJECT TERMS
 Virulence factor, Gram-negative, Francisella, tularemia

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			**19b. TELEPHONE NUMBER** *(include area code)*
U	U	U	UU	58	

14. ABSTRACT

Purpose and Scope: We are developing a novel application of Reverse Phase Protein Microarrays (RPMA) technology to the study of biothreat organisms. The power of this technology to survey the phosphorylation status of multiple proteins simultaneously enables us to map the host cell response to infection with multiple strains and species of Francisella as well as to begin to dissect which individual factors or proteins are contributing to the complex signals generated during infection, and thereby perhaps also to virulence. We will demonstrate the utility of this technology to examine host responses to bacterial infection, host responses to extracellular macromolecules, and host responses to individual proteins applied either extracellularly or intracellularly to the host cell. We will also compare different strains and species of Francisella using RPMA to elucidate the molecular differences in host response to the strains. Furthermore, we will begin to establish a model of how to use RPMAs to screen a genome-worth of open reading frames (ORFs) for potential virulence factors (VFs) by identifying those factors with an effect on host cell signaling pathways.

Results: In the second year of this project, we have completed the majority of Task 1 subtasks, and have continued to make progress on Task 2 subtasks. In the completion of Task 1 subtasks we have performed a thorough validation study of the antibodies used. We have then used the antibodies in an RPMA study of *F. tularensis Live Vaccine Strain, F. tularensis novicida,* and *F. tularensis tularensis NIHB38* infection of J774A.1 murine macrophage or THP-1 human macrophage cells. Purified Lipopolysaccharide (LPS) was applied to host cells and RPMA was performed. We have analyzed the data via unsupervised clustering of heat maps, and Spearman correlation calculations to illustrate linkages between pathways, as part of mapping the interaction of the intracellular pathogen with its host. For Task 2, we have demonstrated the utility of the model of applying bacterial virulence factors to host cells by applying the purified LPS to host cells and performing RPMA. We are ready to apply the first purified bacterial virulence factor protein, IglC, to host cells. We have also either cloned, expressed, or begun cloning multiple other virulence factors of Francisella, in preparation for our work in Year 3.

Significance: We have demonstrated that RPMA can be used to rapidly characterize the effect of bacterial infection on host cells by examining alterations in phosphorylation of multiple signaling pathway proteins. Through the ability of the protein array technology to measure hundreds of signaling events at once, in effect providing a real-time molecular network map, RPMA analysis will allow us to discover the full complement of signaling systems that could serve as new targets for intervention and therapy. The comparative analysis of signaling profiles will further aid us in understanding the key elements of virulence and pathogenesis, a critical step along the path to development of vaccines and therapeutics.

Table of Contents

INTRODUCTION: Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

The age of proteomic technologies has arrived, not just for cancer studies and individualized medicine, but also to the study of microbial pathogenesis of important organisms. Reverse Phase Protein Microarrays (RPMA) are a powerful new technology that can be applied to the post-genomic era of microbial pathogenesis studies. We are developing a novel application of RPMA technology to the study of biothreat organisms – to rapidly characterize the effect of bacterial infection on host cells by examining alterations in phosphorylation of multiple signaling pathway proteins. The power of this technology to survey the phosphorylation status of multiple proteins simultaneously enables us to map the host cell response to infection with multiple strains and species of *Francisella* as well as to begin to dissect which individual factors or proteins are contributing to the complex signals generated during infection, and thereby perhaps also contributing to virulence. We will demonstrate the utility of this technology to examine host responses to bacterial infection, host responses to extracellular macromolecules, and host responses to individual proteins applied either extracellularly or intracellularly to the host cell. Bacterial molecules which exert an effect the host could be considered potential virulence factors, and could be identified as candidates for further study, if not previously known as virulence factors. We will also compare different strains and species of *Francisella* using RPMA to elucidate the molecular differences in host response to the strains. Furthermore, we will begin to establish a model of how to use RPMAs to screen a genome-worth of open reading frames (ORFs) for potential virulence factors (VFs) by identifying those factors with an effect on host cell signaling pathways.

BODY: This section of the report shall describe the research accomplishments associated with each task outlined in the approved Statement of Work. Data presentation shall be comprehensive in providing a complete record of the research findings for the period of the report. Provide data explaining the relationship of the most recent findings with that of previously reported findings. Appended publications and/or presentations may be substituted for detailed descriptions of methodology but must be referenced in the body of the report. If applicable, for each task outlined in the Statement of Work, reference appended publications and/or presentations for details of result findings and tables and/or figures. The report shall include negative as well as positive findings. Include problems in accomplishing any of the tasks. Statistical tests of significance shall be applied to all data whenever possible. Figures and graphs referenced in the text may be embedded in the text or appended. Figures and graphs can also be referenced in the text and appended to a publication. Recommended changes or future work to better address the research topic may also be included, although changes to the original Statement of Work must be approved by the Army Contracting Officer Representative. This approval must be obtained prior to initiating any change to the original Statement of Work.

Summary of Tasks: Specifically, this project includes the following tasks.

Task 1: Validation of RPMA technology

1. Generate the phosphoactivation map of *Francisella LVS* infected J774A.1 macrophage cells.
2. Generate the phosphoactivation map of *Francisella LVS* Lipopolysaccharide (LPS) treated J774A.1 macrophage cells.
3. Compare the phosphoactivation profiles generated by RPMA to published results in J774A.1 cells and provide conclusions regarding the validation of RPMA technology.
4. Generate the phosphoactivation map of *Francisella LVS* infected human activated THP-1 (macrophage) cells. Compare the phosphoactivation profiles between J774A.1 and THP-1 cells. Provide conclusions regarding the differences in response of human and mouse cells to *Francisella LVS* infection.
5. Generate the phosphoactivation map of *Francisella tularensis B38* infected J774A.1 macrophage cells. Compare the phosphoactivation profiles generated to published results with other Francisella strains in J774A.1 cells for validation.
6. Analyze and compare the phosphoactivation profiles generated by the type strain B38 to vaccine (LVS) strain. Provide conclusions regarding the differences between the strains of Francisella.
7. Generate the phosphoactivation map of *Francisella tularensis novicida* infected J774A.1 macrophage cells. Compare the phosphoactivation profiles generated to published results with other *Francisella* strains in J774A.1 cells for validation.
8. Analyze and compare the phosphoactivation profiles generated by *F. novicida* to vaccine (*LVS*) strain. Provide conclusions regarding the differences between the strains of *Francisella*.

Task 2: Screening of *Francisella* ORFs for potential VFs as a model for high-throughput screening of ORFs.

1. Clone and express a set of *Francisella* proteins, including *F. tularensis, F. novicida and F. tularensis LVS* genes as available (and where they are significantly different that the Ft genes). Confirm activities (if known) of expressed proteins vs. native purified proteins.
2. Apply *Francisella* proteins extracellularly to mouse (J774A.1 macrophages) and human (activated THP-1) cells, and then generate the phosphoactivation map.
3. Transfect plasmids expressing *Francisella* proteins into mouse (J774A.1 macrophages) and human (activated THP-1) cells, confirm intracellular expression, and then generate the phosphoactivation map.

Task 1: Validation of Reverse Phase Protein Microarray (RPMA) technology

A. Validation of Antibodies used in RPMA analysis:

This task was assumed in the specific tasks, but is called out here for presentation purposes. The antibodies used in our studied have been validated in the A431 (Human epithelial carcinoma cell line) Whole Cell Lysate prior to use by the laboratory of Drs. Liotta and

Petricoin. However, based on some of our results indicating that the validation may be cell line specific, we returned to this issue to re-validate all the antibodies we use in the J774A.1 murine macrophage cell line.

The criteria for validation of the antibody for use in RPMA is that it produces one predominant band when used in Western Blotting with the particular cell lysate of interest. Or, if multiple bands are present, that they are all specific isoforms of the protein and not non-specific bands. Thus, we have performed validation studies for the following antibodies to date:

Figure 1A: Validation Assay For phospho NFkB.
Western blot of phospho NFkB (cell signaling 3031) (65 kDa) total protein concentration in infected J774A.1 cells by *Francisella novicida.* Briefly, J774A.1 cells were plated in a 6 well plate 1 day prior to infection. Cells were either infected with 1* 10^6 *Francisella novicida* or an equal amount of media. Pre-infection was performed for 2 hours prior to the addition of gentamycin solution I. Gentamycin solution I was left in the wells for 1 hour to ensure that extacellular bacteria were lysed. Time 0 was then calculated by the point when the gentamycin solution 2 was added. All cells were lysed at the same time. Cells were then lysed 4 hours after t=0. *(Refer to p.5 for full infection protocol. Results of protein concentration p.94-97, 107.)* Lane 1 is Magic Mark XP western ladder. Lane 2 is non-infected control J774A.1 cells. Lane 3 is J774A.1 cells infected with *F. novicida* when time = 4 hours. **Although the blot is overexposed to check for additional bands, it can be seen that there is one dominant band. The antibody is scored as a pass.**
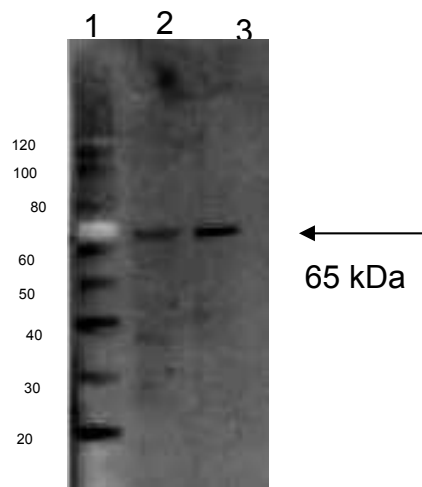
Figure 1B: Validation Assay For NFkB. Western blot of NFkB (cell signaling 3034) (65 kDa) total protein concentration in infected J774A.1 cells by *Francisella novicida.* Briefly, J774A.1 cells were plated in a 6 well plate 1 day prior to infection. Cells were either infected with 1* 10^6 *Francisella novicida* or an equal amount of media. Pre-infection was performed for 2 hours prior to the addition of gentamycin solution I. Gentamycin solution I was left in the wells for 1 hour to ensure that extacellular bacteria were lysed. Time 0 was then calculated by the point when the gentamycin solution 2 was added. All cells were lysed at the same time. Cells were then lysed 4 hours after t=0. *(Refer to p.5 for full infection protocol. Results of protein concentration p.94-97, 107.)* Lane 1 is ez-run pre stained rec protein ladder (fisher BP3603-500) western ladder. Lane 2 is non-infected control J774A.1 cells. Lane 3 is J774A.1 cells infected with *F. novicida* when time = 4 hours. **This blot is presented as overexposed to observe backgound bands, but the dominant band upon lighter exposure in NFKB, and so this antibody is scored as pass.**
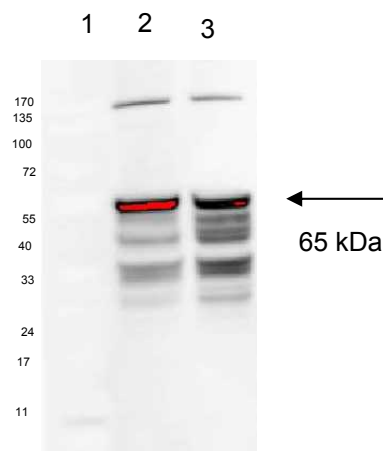
Figure 1C: Western blot of **AKT** (Cell Signaling 4691) (60 kDa) in infected J774A.1 cells by *Francisella novicida.* Briefly, J774A.1 cells were plated in a 6 well plate 1 day prior to infection. Cells were either infected with 1* 10^6 *Francisella novicida* or an equal amount of media. Pre-infection was performed for 2 hours prior to the addition of gentamycin solution I. Gentamycin solution I was left in the wells for 1 hour to ensure that extacellular bacteria were lysed. Time 0 was then calculated by the point when the gentamycin solution 2 was added. All cells were lysed at the same time. Cells were then lysed 4 hours after t=0. *(Refer to p.5 for full infection protocol. Results of protein concentration p.94-97, 107.)* Lane 1 is Magic Mark XP western ladder. Lane 2 is non-infected control J774A.1 cells. Lane 3 is J774A.1 cells infected with *F. novicida* when time = 4 hours.

Figure 1D: Western blot of **phospho-AKT Thr308** (Cell Signaling 2965) (60 kDa) in infected J774A.1 cells by *Francisella novicida.* Briefly, J774A.1 cells were plated in a 6 well plate 1 day prior to infection. Cells were either infected with 1* 10^6 *Francisella novicida* or an equal amount of media. Pre-infection was performed for 2 hours prior to the addition of gentamycin solution I. Gentamycin solution I was left in the wells for 1 hour to ensure that extacellular bacteria were lysed. Time 0 was then calculated by the point when the gentamycin solution 2 was added. All cells were lysed at the same time. Cells were then lysed 4 hours after t=0. *(Refer to p.5 for full infection protocol. Results of protein concentration p.94-97, 107. Picture came from validation 9-18-07)* Lane 1 is Magic Mark XP western ladder. Lane 2 is non-infected control J774A.1 cells. Lane 3 is J774A.1 cells infected with *F. novicida* when time = 4 hours.
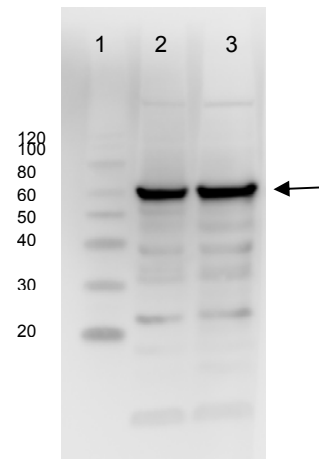

Figure 1E: Western blot of **Caspase 1** (Cell Signaling 2225) (p20 20 kDa, β,δ, γ 30-45 kDa α 50 kDa) in infected J774A.1 cells by *Francisella novicida.* Briefly, J774A.1 cells were plated in a 6 well plate 1 day prior to infection. Cells were either infected with 1* 10^6 *Francisella novicida* or an equal amount of media. Pre-infection was performed for 2 hours prior to the addition of gentamycin solution I. Gentamycin solution I was left in the wells for 1 hour to ensure that extacellular bacteria were lysed. Time 0 was then calculated by the point when the gentamycin solution 2 was added. All cells were lysed at the same time. Cells were then lysed 4 hours after t=0. *(Refer to p.5 for full*

infection protocol. Results of protein concentration p.94-97, 107. Picture came from validation 9-18-07) Lane 1 is Magic Mark XP western ladder. Lane 2 is non-infected control J774A.1 cells. Lane 3 is J774A.1 cells infected w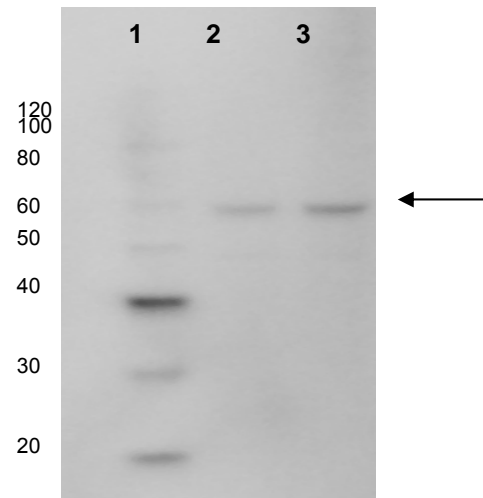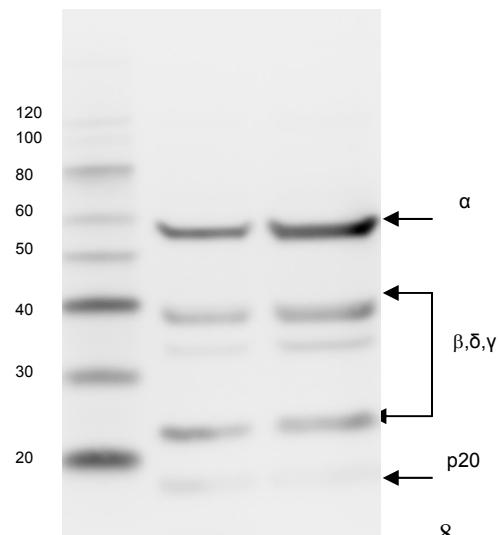ith *F. novicida* when time = 4 hours. **Although it has multiple bands, each band can be accounted for by an isoform of Caspase 1, so the antibody is scored as a pass.**

Figure 1F: Western blot of **phospho- p44/42 Map Kinase** (Cell Signaling 4370) (p44 44 kDa, p42 42 kDa) in infected J774A.1 cells by *Francisella novicida.* Briefly, J774A.1 cells were plated in a 6 well plate 1 day prior to infection. Cells were either infected with 1* 10^6 *Francisella novicida* or an equal amount of media. Pre-infection was performed for 2 hours prior to the addition of gentamycin solution I. Gentamycin solution I was left in the wells for 1 hour to ensure that extacellular bacteria were lysed. Time 0 was then calculated by the point when the gentamycin solution 2 was added. All cells were lysed at the same time. Cells were then lysed 4 hours after t=0. *(Refer to p.5 for full infection protocol. Results of protein concentration p.94-97, 107. Picture came from validation 9-18-07)* Lane 1 is Magic Mark XP western ladder. Lane 2 is non-infected control J774A.1 cells. Lane 3 is J774A.1 cells infected with *F. novicida* when time = 4 hours.

Figure 1G: Western blot of **total p44/42 Map Kinase** (Cell Signaling 4695) (p44 44 kDa, p42 42 kDa) total protein concentration in infected J774A.1 cells by Francisella novicida. Briefly, J774A.1 cells were plated in a 6 well plate 1 day prior to infection. Cells were either infected with 1* 10^6 Francisella novicida or an equal amount of media. Pre-infection was performed for 2 hours prior to the addition of gentamycin solution I. Gentamycin solution I was left in the wells for 1 hour to ensure that extacellular bacteria were lysed. Time 0 was then calculated by the point when the gentamycin solution 2 was added. All cells were lysed at the same time. Cells were then lysed 4 hours after t=0. (Refer to p.5 for full infection protocol. Results of protein concentration p.94-97, 107. Picture came from validation 9-18-07) Lane 1 is Magic Mark XP western ladder. Lane 2 is non-infected control J774A.1 cells. Lane 3 is J774A.1 cells infected with F. novicida when time = 4 hours.
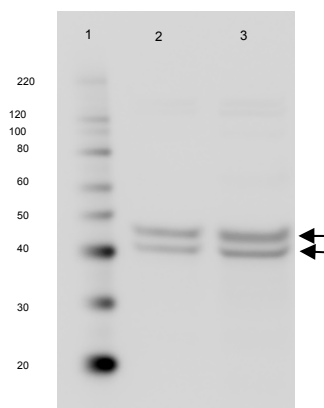
Figure 1H: Total MKK3 Validation blot. Western blot of MKK3 (Cell Signaling 9232) (40 kDa) total protein concentration in infected J774A.1 cells by Francisella novicida. Briefly, J774A.1 cells were plated in a 6 well plate 1 day prior to infection. Cells were either infected with 1* 10^6 Francisella novicida or an equal amount of media. Pre-infection was performed for 2 hours prior to the addition of gentamycin solution I. Gentamycin solution I was left in the wells for 1 hour to ensure that extacellular bacteria were 9232 lysed. Time 0 was then calculated by the point when the gentamycin solution 2 was added. All cells were lysed at the same time. Cells were then lysed 4 hours after t=0. (Refer to p.5 for full infection protocol. Results of protein concentration p.94-97, 107. Picture

came from validation 9-18-07) Lane 1 is EZ-Run pre stained Rec protein ladder (Fisher BP3603-500). Lane 2 is non-infected control J774A.1 cells. Lane 3 is J774A.1 cells infected with F. novicida when time = 4 hours. **The white bands represent the high concentration of rainbow molecule weight markers that are also run in the MW marker lane for visualization during transfer. The blot is overexposed to see background bands, but the dominant band is MKK3 and so the antibody is scored as pass.**

Figure 1I: Example of a protein that did NOT pass validation. Western blot of phospho **MKK3** (Cell Signaling 9230) (40 kDa)   in infected J774A.1 cells by *Francisella novicida.* Briefly, J774A.1 cells were plated in a 6 well plate 1 day prior to infection. Cells were either infected with 1* 10^6 *Francisella novicida* or an equal amount of media. Pre-infection was performed for 2 hours prior to the addition of gentamycin solution I. Gentamycin solution I was left in the wells for 1 hour to ensure that extacellular bacteria were lysed. Time 0 was then calculated by the point when the gentamycin solution 2 was added. All cells were lysed at the same time. Cells were then lysed 4 hours after t=0. *(Refer to p.5 for full infection protocol. Results of protein concentration p.94-97, 107. Picture came from validation 9-18-07)*   Lane 1 is Magic Mark XP western ladder. Lane 2 is non-infected control J774A.1 cells.

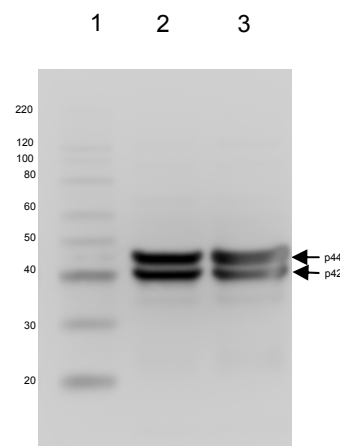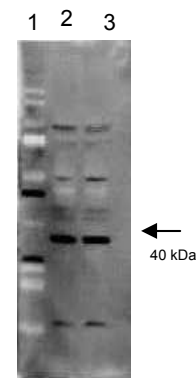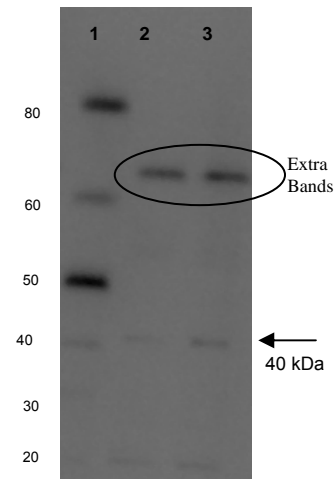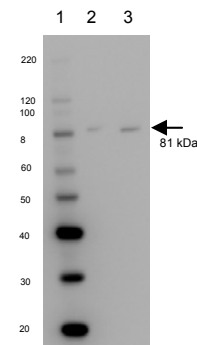Lane 3 is J774A.1 cells infected with *F. novicida* when time = 4 hours. **In this blot, the dominant band is of a much higher MW than the phosphorylated form of MKK3. This result was observed with three independent sets of lysates and blots and so the antibody is scored as a Fail.**

Figure 1J: Validation Assay For IRAK: Western blot of **IRAK** (Cell Signaling 4362) (81 kDa)   in infected J774A.1 cells by *Francisella novicida.* Briefly, J774A.1 cells were plated in a 6 well plate 1 day prior to infection. Cells were either infected with 1* 10^6 *Francisella novicida* or an equal amount of media. Pre-infection was performed for 2 hours prior to the addition of gentamycin solution I. Gentamycin solution I was left in the wells for 1 hour to ensure that extacellular bacteria were lysed. Time 0 was then calculated by the point when the gentamycin solution 2 was added. All cells were lysed at the same time. Cells were then lysed 4 hours after t=0. *(Refer to p.5 for full infection protocol. Results of protein concentration p.94-97, 107. Picture came from validation 9-18-07).* Lane 1 is Magic Mark XP western ladder. Lane 2 is non-infected control J774A.1 cells. Lane 3 is J774A.1 cells infected with *F. novicida* when time = 4 hours.

Figure 1K: **Phospho-Bad (Ser112) Antibody**: Western blot of Bad (Cell Signaling 9291) (25 kDa)   in infected J774A.1 cells by *Francisella novicida.* Briefly, J774A.1 cells were plated in a 6 well plate 1 day prior to infection. Cells were either infected with 1* 10^6 *Francisella novicida* or an equal amount of media. Centrifuge plates at 50g, 4 degree for five minutes after adding media or bacteria. Incubate in 37 degree incubator for 5minute prior to the addition of gentamycin solution I (50ug/ml gentamycin). Wash with gentamycin solution I briefly, add getamycin solution 2 (2ug/ml gentamycin), incubate in a 37 degree incubator (start counting up right after

plates were taken out of the centrifuge). Cells were then lysed 4 hours after. Lane 1, Magic MarkXP; lane 2 is EGF-stimulated A431; lane 3 is J774A.1 cells infected with *F. novicida* when time = 4 hours. **Although the band is very light, it is a single band, and this antibody is scored as a pass.**
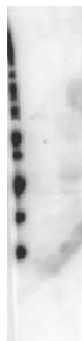
Figure 1L: **Phospho-p38 MAP Kinase** (Thr180/Tyr182) Antibody: Western blot of Bad (Cell Signaling 9211) (43 kDa) in infected J774A.1 cells by *Francisella novicida.* Briefly, J774A.1 cells were plated in a 6 well plate 1 day prior to infection. Cells were either infected with 1* 10^6 *Francisella novicida* or an equal amount of media. Centrifuge plates at 50g, 4 degree for five minutes after adding media or bacteria. Incubate in 37 degree incubator for 5minute prior to the addition of gentamycin solution I (50ug/ml gentamycin). Wash with gentamycin solution I briefly, add getamycin solution 2 (2ug/ml gentamycin), incubate in a 37 degree incubator (start counting up right after plates were taken out of the centrifuge). Cells were then lysed 4 hours after. Lane 1, Magic MarkXP; lane 2 is EGF-stimulated A431; lane 3 is J774A.1 cells infected with *F. novicida* when time = 4 hours.

Phospho-p38 MAPK (Thr180/Tyr182)

Figure 1M: **Cleaved Caspase-9 (Asp315) Antibody** (Human Specific): Western blot of CC9 (Cell Signaling 9505) (35 kDa) in infected J774A.1 cells by *Francisella novicida.* Briefly, J774A.1 cells were plated in a 6 well plate 1 day prior to infection. Cells were either infected with 1* 10^6 *Francisella novicida* or an equal amount of media. Centrifuge plates at 50g, 4 degree for five minutes after adding media or bacteria. Incubate in 37 degree incubator for 5minute prior to the addition of gentamycin solution I (50ug/ml gentamycin). Wash with gentamycin solution I briefly, add getamycin solution 2 (2ug/ml gentamycin), incubate in a 37 degree incubator (start counting up right after plates were taken out of the centrifuge). Cells were then lysed 4 hours after. Lane 1, Magic MarkXP; lane 2 is EGF-stimulated A431; lane 3 is J774A.1 cells infected with *F. novicida* when time = 4 hours.

Caspas 9

Figure 1N: **Phospho-IkB-a (Ser32/36) (5A5) Mouse** mAb: Western blot of Phospho-IkBa (Cell Signaling 9246) (40 kDa) in infected J774A.1 cells by *Francisella novicida.* Briefly, J774A.1 cells were plated in a 6 well plate 1 day prior to infection. Cells were either infected with 1* 10^6 *Francisella novicida* or an equal amount of media. Centrifuge plates at 50g, 4 degree for five minutes after adding media or bacteria. Incubate in 37 degree incubator for 5minute prior to the addition of gentamycin solution I (50ug/ml gentamycin). Wash with gentamycin solution I briefly, add getamycin solution 2 (2ug/ml gentamycin), incubate in a 37 degree incubator (start counting up right after plates were taken out of the centrifuge). Cells were then lysed 4 hours after. Lane 1, Magic MarkXP; lane 2 is EGF-stimulated A431; lane 3 is J774A.1 cells infected with *F. novicida* when time = 4 hours.
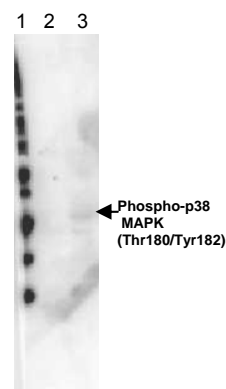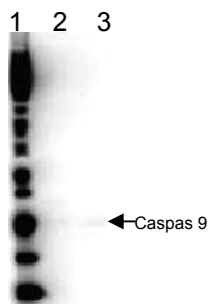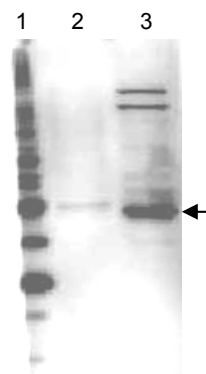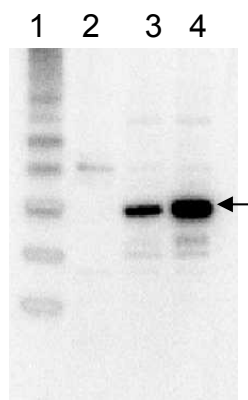
1 2 3 4

Figure 1O: **Phospho-Bcl-2 (Ser70) (5H2) Rabbit mAb**: Western blot of Phospho-Bcl-2 (Cell Signaling 2827) (28 kDa) total protein concentration in infected J774A.1 cells by *Francisella novicida.* Briefly, J774A.1 cells were plated in a 6 well plate 1 day prior to infection. Cells were either infected with 1* 10^6 *Francisella novicida* or an equal amount of media. Centrifuge plates at 50g, 4 degree for five minutes after adding media or bacteria. Incubate in 37 degree incubator for 5minute prior to the addition of gentamycin solution I (50ug/ml gentamycin). Wash with gentamycin solution I briefly, add getamycin solution 2 (2ug/ml gentamycin), incubate in a 37 degree incubator (start counting up right after plates were taken out of the centrifuge). Cells were then lysed 4 hours after. Lane 1, Magic MarkXP; lane 2 is EGF-stimulated A431; lane 3 is control J774A.1 cells; lane 4 is J774A.1 cells infected with *F. novicida* when time = 4 hours.

Figure 1P: **Phospho-SAPK/JNK (Thr183/Tyr185) Antibody**. Western blot of Phospho-SAPK/JNK (Thr183/Tyr185) Antibody (Cell Signaling 9251) (46 kDa (Phospho-JNK1), 54 kDa (Phospho-JNK2/3) with total protein concentration in infected J774A.1 cells by *Francisella novicida.* Briefly, J774A.1 cells were plated in a 6 well plate 1 day prior to infection. Cells were either infected with 1* 10^6 *Francisella novicida* or an equal amount of media. Centrifuge plates at 50g, 4 degree for five minutes after adding media or bacteria. Incubate in 37 degree incubator for 5minute prior to the addition of gentamycin solution I (50ug/ml gentamycin). Wash with gentamycin solution I briefly, add getamycin solution 2 (2ug/ml gentamycin), incubate in a 37 degree incubator (start counting up right after plates were taken out of the centrifuge). Cells were then lysed 4 hours after. Lane 1, Magic MarkXP; lane 2 is EGF-stimulated A431; lane 3 is control J774A.1 cells; lane 4 is J774A.1 cells infected with *F. novicida* when time = 4 hours.

1 2 3 4

Phospho-p54

Phospho-p46

Figure 1Q: **Bax Antibody.** Western blot of **Bax Antibody** (Cell Signaling 2772) (20kDa) total protein concentration in infected J774A.1 cells by *Francisella novicida.* Briefly, J774A.1 cells were plated in a 6 well plate 1 day prior to infection. Cells were either infected with 1* 10^6 *Francisella novicida* or an equal amount of media. Centrifuge plates at 50g, 4 degree for five minutes after adding media or bacteria. Incubate in 37 degree incubator for 5minute prior to the addition of gentamycin solution I (50ug/ml gentamycin). Wash with gentamycin solution I briefly, add getamycin solution 2 (2ug/ml gentamycin), incubate in a 37 degree incubator (start counting up right after plates were taken out of the centrifuge). Cells were then lysed 4 hours after. Lane 1, Magic MarkXP; lane 2 is EGF-stimulated A431; lane 3 is control J774A.1 cells; lane 4 is J774A.1 cells infected with *F. novicida* when time = 4 hours.

1 2 3 4

◄ Ba

Figure 1R: **Phospho-Paxillin (Tyr118) Antibody** (2541). Western blot of Phospho-Paxillin (Tyr118) Antibody (Cell Signaling 2541) (68 kDa) total protein concentration in infected J774A.1 cells by *Francisella novicida.* Briefly, J774A.1 cells were plated in a 6 well plate 1 day prior to infection. Cells were either infected with 1* 10^6 *Francisella novicida* or an equal amount of media. Centrifuge plates at 50g, 4 degree for five minutes after adding media or bacteria. Incubate in 37 degree incubator for 5minute prior to the addition of gentamycin solution I (50ug/ml gentamycin). Wash with gentamycin solution I briefly, add getamycin solution 2 (2ug/ml gentamycin), incubate in a 37 degree incubator (start counting up right after plates were taken out of the centrifuge). Cells were then lysed 4 hours after.   Lane 1, Magic MarkXP; lane 2 is EGF-stimulated A431; lane 3 is control J774A.1 cells; lane 4 is J774A.1 cells infected with *F. novicida* when time = 4 hours.
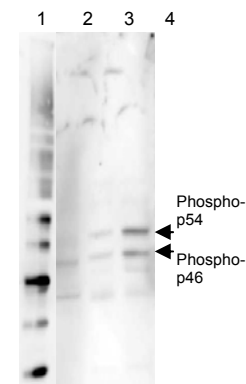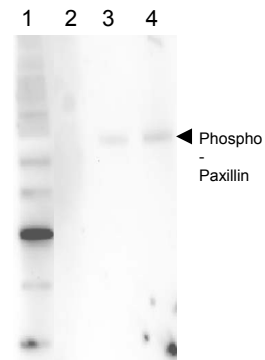
Figure 1S: **Another example of a failed antibody**. **Phospho-Pyk2 (Tyr402) Antibody (3291**). Western blot of Phospho-Pyk2 Antibody (Tyr402) Antibody (Cell Signaling 3291) (116 kDa) total protein concentration in infected J774A.1 cells by *Francisella novicida.* Briefly, J774A.1 cells were plated in a 6 well plate 1 day prior to infection. Cells were either infected with 1* 10^6 *Francisella novicida* or an equal amount of media. Centrifuge plates at 50g, 4 degree for five minutes after adding media or bacteria. Incubate in 37 degree incubator for 5minute prior to the addition of gentamycin solution I (50ug/ml gentamycin). Wash with gentamycin solution I briefly, add getamycin solution 2 (2ug/ml gentamycin), incubate in a 37 degree incubator (start counting up right after plates were taken out of the centrifuge). Cells were then lysed 4 hours after.   Lane 1, Magic MarkXP; lane 2 is EGF-stimulated A431; lane 3 is J774A.1 cells infected with *F. novicida* when time = 4 hours. **This antibody did not recognize phospho-Paxillin asn the dominant band on the gel when tested on murine macrophage lysate in one blot. Thus, this antibody was scored as a probable fail. We are repeating this experiment to confirm its fail status. However, it might still work fine with human THP-1 lysate, so this needs to be tested.**
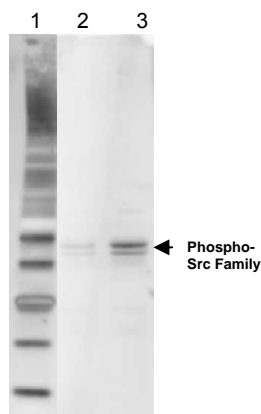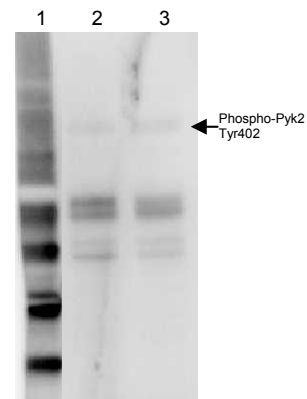
Figure 1T: **Phospho-Src Family (Tyr416) Antibody (2101).** Western blot of Phospho-Src Family (Tyr416) Antibody (Cell Signaling 2101) (57 kDa) total protein concentration in infected J774A.1 cells by Francisella novicida. Briefly, J774A.1 cells were plated in a 6 well plate 1 day prior to infection. Cells were either infected with 1* 10^6 Francisella novicida or an equal amount of media. Centrifuge plates at 50g, 4 degree for five minutes after adding media or bacteria. Incubate in 37 degree incubator for 5minute prior to the addition of gentamycin solution I (50ug/ml gentamycin). Wash with gentamycin solution I briefly, add getamycin solution 2 (2ug/ml gentamycin), incubate in a 37 degree incubator (start counting up right after plates were taken out of the centrifuge). Cells were then lysed 4 hours after.   Lane 1, Magic MarkXP; lane 2 is EGF-stimulated A431; lane 3 is J774A.1 cells infected with F. novicida when time = 4 hours.

**Remaining antibodies to Validate**: Bad S155, Lck, FAK, phospho-Pyk2 (repeat), Caspase 3, phospho-Pyk2 and phosphoMKK3 with THP-1 lysate.

Summary Table:

Figure	Antibody	Validated/Passed in J774A.1 murine macrophages
1A	Phospho-NFKB	Pass
1B	Total NFKB	Pass
1C	Total AKT (Cell Signaling 4691)	Pass
1D	Phospho-AKT Thr 308 (Cell Signaling 2965)	Pass
1E	Caspase 1 (Cell Signaling 2225)	Multiple Isoforms - Validated
1F	Phospho-P42/p44 MAPK (Cell Signaling 4370)	Two Isoforms - Validated
1G	Total p44/42 Map Kinase (Cell Signaling 4695)	Two Isoforms - Validated
1H	Total MKK3 (Cell Signaling 9232)	Pass
1I	Phospho-MKK3	**FAIL**
1J	Total IRAK (Cell Signaling 4362)	Pass
1K	pBAD-S112	Pass
1L	Phospho-p38	Pass
1M	Cleaved Caspase 9	Pass
1N	Phospho-ikBa	Pass
1O	Phospho Bcl2	Pass
1P	Phospho-SAP/Jnk	Pass
1Q	Bax	Pass
1R	Phospho-Paxillin	Pass
1S	Phospho-Pyk2	**Probable FAIL**
1T	Phospho-Src	Pass

Conclusion: In conclusion, some antibodies, such as the antibody for phospho MKK3 and phospho Pyk2 can not be validated in J774A.1 lysates and thus can not be used in RPMA from J774A.1 cells. They may be human specific antibodies and poorly reactive in the mouse system. These antibodies may be validated in THP-1 cells, in which case they can be used for that cell line. Other antibodies, such as the p42/p44 MAP kinase antibodies are fine and can be used in RPMA from J774A.1 cells. These results emphasize the importance of careful controls when doing RPMA experiments.

Task 1.1: Generate the phosphoactivation map of *Francisella* LVS infected J774A.1 macrophage cells.

Murine macrophages were infected with Francisella tularensis Live Vaccine Strain (Francisella LVS) for various times. Lysates were prepared and applied to Whatman Fast Slides robotically. The slides were then incubated with the indicated antibody solutions and antibody binding was detected by scanning stained slides. Analysis of the dilution series was performed as discussed in detail in the previous Annual Report. Data were analyzed to pick the optimal dilution. Changes in antibody binding are presented on the following graphs. This general method was followed for all the RPMA experiment presented here.

Figure 1.1A: Activation profile of *Francisella tularensis* LVS infected J774A.1 macrophages.

Activation profile of F. LVS infected J774A.1 macrophages
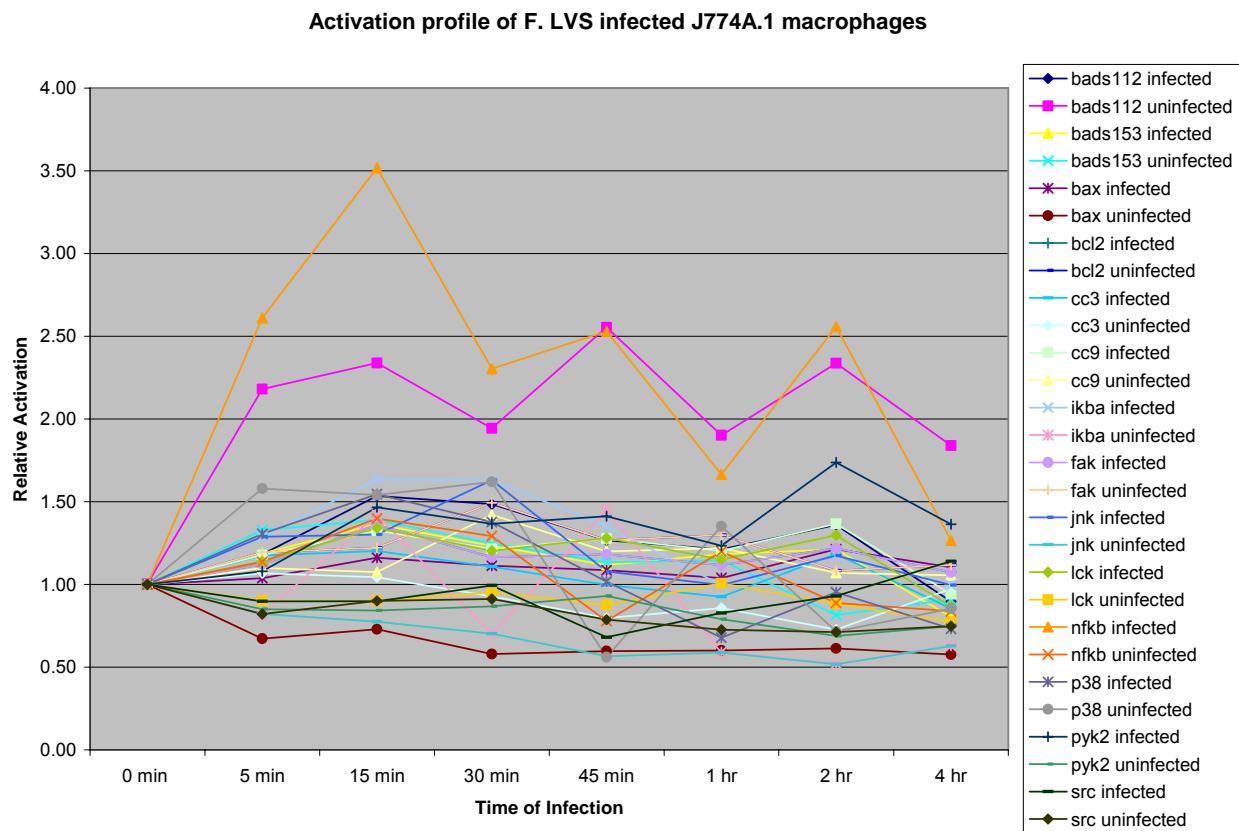
This graph (Figure 1.1A) represents the RPMA data for both uninfected and infected cells at the various time of the experiment. Time zero was set to one for each data set for easy comparison. These data are also summarized in the Table 1.1A below.

Table 1.1A: The relative changes detected by RPMA are summarized in the Table below for Uninfected and LVS infected J774A.1 cells.

	Uninfected								LVS Infected							
time in minutes	0	5	15	30	45	60	120	240	0	5	15	30	45	60	120	240
bads112	1.00	2.18	2.34	1.94	2.55	1.90	2.34	1.84	1.00	1.18	1.54	1.49	1.27	1.21	1.36	0.90
bads153	1.00	1.33	1.39	1.25	1.13	1.14	0.82	0.91	1.00	1.19	1.36	1.23	1.12	1.18	1.21	0.80
bax	1.00	0.67	0.73	0.58	0.60	0.60	0.61	0.58	1.00	1.04	1.16	1.11	1.09	1.04	1.21	1.10
bcl2	1.00	1.19	1.22	1.49	1.27	1.30	1.08	1.12	1.00	1.12	1.36	1.16	1.18	1.12	1.22	1.07
Caspase 3	1.00	1.07	1.04	0.93	0.80	0.86	0.73	0.97	1.00	1.18	1.20	1.11	1.00	0.93	1.18	0.85
Caspase 9	1.00	1.10	1.07	1.42	1.20	1.22	1.07	1.06	1.00	1.18	1.32	1.21	1.28	1.20	1.37	0.94
IkappaBalpha	1.00	0.82	1.42	0.70	1.45	0.59	0.52	0.63	1.00	1.29	1.64	1.63	1.35	1.00	1.17	1.00
fak	1.00	0.89	0.88	0.74	0.86	0.89	0.86	0.73	1.00	1.07	1.34	1.11	0.87	0.93	0.96	0.90
jnk	1.00	0.82	0.77	0.70	0.57	0.59	0.52	0.63	1.00	1.29	1.30	1.63	1.08	1.00	1.17	1.00
lck	1.00	0.90	0.90	0.95	0.88	1.01	0.88	0.77	1.00	1.14	1.34	1.20	1.28	1.15	1.30	0.87
nfkb	1.00	1.14	1.40	1.29	0.78	1.20	0.89	0.84	1.00	2.61	3.52	2.30	2.53	1.67	2.56	1.27
p38	1.00	1.58	1.54	1.62	0.56	1.35	0.72	0.85	1.00	1.31	1.55	1.38	1.02	0.68	0.95	0.73
pyk2	1.00	0.85	0.84	0.87	0.93	0.79	0.69	0.75	1.00	1.08	1.47	1.37	1.41	1.24	1.74	1.36
src	1.00	0.82	0.90	0.91	0.79	0.73	0.71	0.75	1.00	0.90	0.90	0.99	0.68	0.83	0.93	1.14

Figure 1.1B: Relative Changes in Activation of *Francisella tularensis* LVS infected vs uninfected J774A.1 macrophages.

In this figure, the infected numbers were divided by the uninfected numbers to illustrate the fold change in signal for each antibody. This representation of fold-change simplifies the data set and allows up to observe which molecules are significantly up-regulated (greater than 1.5) or down-regulated (less than 0.5) following infection by Francisella LVS.
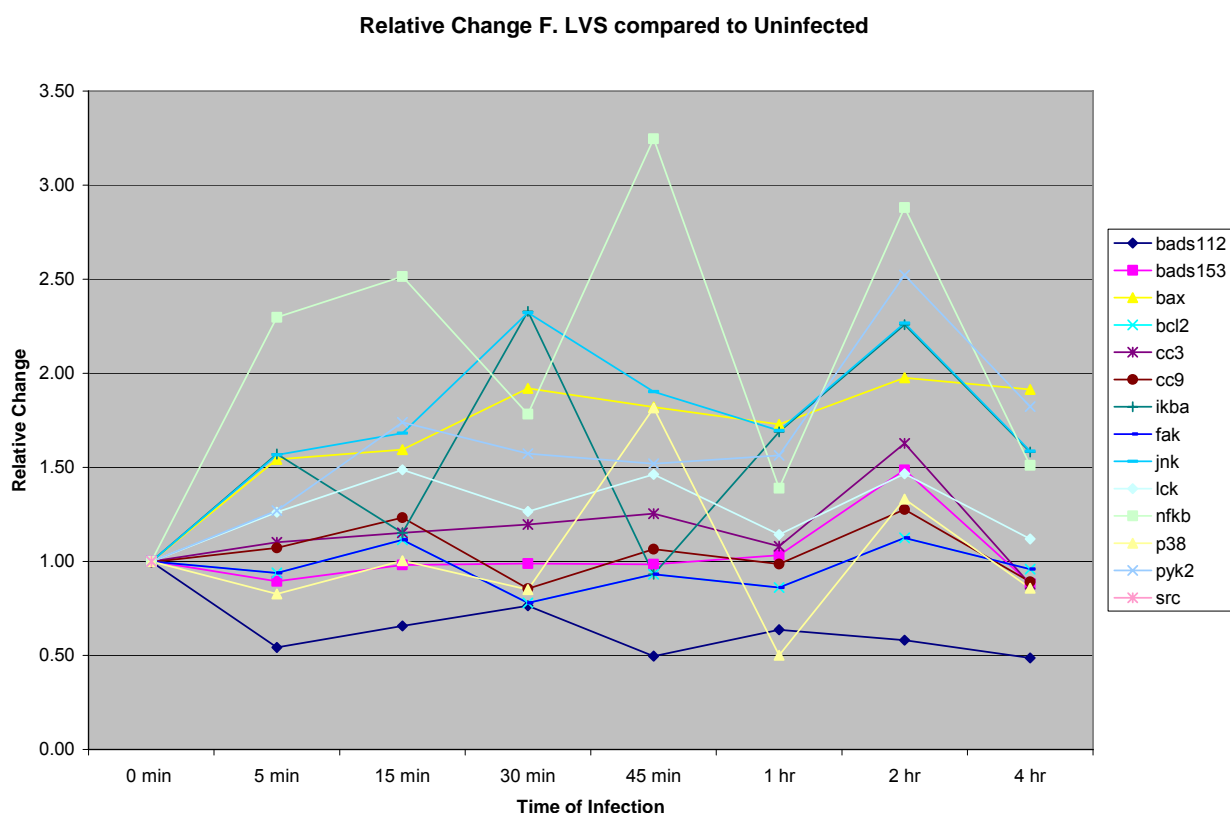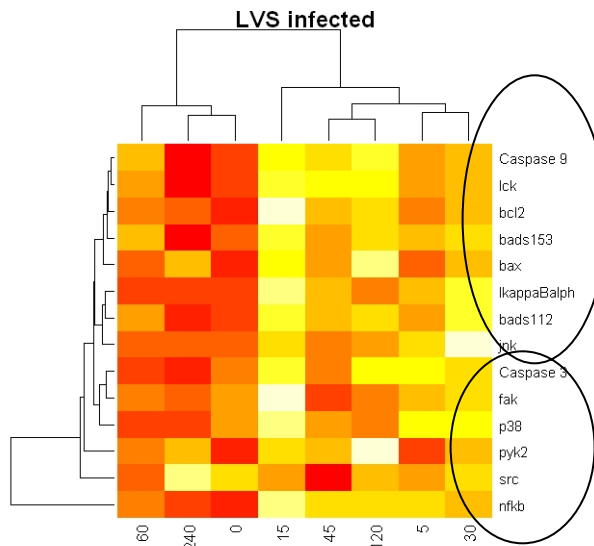

Relative Change F. LVS compared to Uninfected

Figure 1.1C: Heat Map. This data set represents unsupervised clustering of the LVS infection data represented in Table 1.1A. This approach allows the computer to group the data

according to the samples that are most similar. This approach may reveal clustering of signalling pathways, as has been published for other experiments using RPMA (Espina et al, 2004, Liotta et al, 2003). In this case, it can be seen that there is generally clustering of the apopotsis related molecules, Caspase 9, bcl-2, Bax, BadS153, Caspase 3, and BadS112. The second cluster includes p38, Pyk2 (which has to be excluded, see validation above), src, and Fak.

Spearman Correlation analysis looks for molecules that have similar patterns of change over the time course of the experiment (Kotlyar et al 2002). Thus, molecules which have significant p-values are likely to change in the same general way in the experiment (Table 1.1B). Molecules which have significant p-values also have high Rho values, and are likely to change in the same general way in the experiment (Table 1.1C). This may indicate that these molecules are on the same pathway as each other. Francisella LVS infection is relatively activating, and many pairs of molecules are significantly (p>0.05) changing in correlation with each other. Thus, BadS112 and BadS153 are correlated, both by p-value and by rho coefficient. Caspase 9 is correlated to Bad, and Bcl2.

Table 1.1B: Spearman Rho Correlation calculation: In this table, the p-values of the SpearmanRho correlation calculation are presented.

P-value	F LVS													
	bads112	bads153	bax	bcl2	Caspase 3	Caspase 9	IkappaBalpha	fak	jnk	lck	nfkb	p38	pyk2	src
bads112	4.96E-05	0.004563	0.069395	0.004563	0.0575893	0.0072421	0.015376984	0.196627	0.027927	0.002232	0.083085	0.11498	0.045833	0.326835
bads153	NA	4.96E-05	0.11498	0.027927	0.0107143	0.0458333	0.027926587	0.036756	0.007242	0.027927	0.036756	0.083085	0.170982	0.500794
bax	NA	NA	4.96E-05	0.027927	0.2430556	0.0575893	0.196626984	0.582143	0.151141	0.069395	0.196627	0.500794	0.002232	0.840129
bcl2	NA	NA	NA	4.96E-05	0.0575893	0.0011409	0.027926587	0.500794	0.083085	0.000397	0.021776	0.216171	0.004563	0.216171
Caspase 3	NA	NA	NA	NA	4.96E-05	0.0830853	0.045833333	0.036756	0.021776	0.057589	0.010714	0.036756	0.26746	0.619097
Caspase 9	NA	NA	NA	NA	NA	4.96E-05	0.096180556	0.619097	0.151141	0.000397	0.069395	0.389385	0.010714	0.216171
IkappaBalpha	NA	NA	NA	NA	NA	NA	4.96E-05	0.151141	0.004563	0.045833	0.036756	0.004563	0.151141	0.389385
fak	NA	NA	NA	NA	NA	NA	NA	4.96E-05	0.045833	0.461806	0.26746	0.045833	0.976786	0.703323
jnk	NA	NA	NA	NA	NA	NA	NA	NA	4.96E-05	0.11498	0.045833	0.010714	0.243056	0.752034
lck	NA	NA	NA	NA	NA	NA	NA	NA	NA	4.96E-05	0.045833	0.243056	0.015377	0.170982
nfkb	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	4.96E-05	0.11498	0.151141	0.196627
p38	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	4.96E-05	0.582143	0.793006
pyk2	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	4.96E-05	0.582143
src	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	4.96E-05

Table 1.1C: Spearman Rho Correlation calculation: In this table, the Rho correlation of the SpearmanRho correlation calculation are presented. All pairs with significant P-values in the Table above have significant Rho correlations in the Table below.

F LVS
Rho (estimate of correlation)

	bads112	bads153	bax	bcl2	Caspase 3	Caspase 9	IkappaBalpha	fak	jnk	lck	nfkb	p38	pyk2	src
bads112	1	0.904762	0.682647	0.89822	0.6988459	0.8809524	0.829515062	0.52381	0.78072	0.928571	0.666667	0.619048	0.738095	-0.395217
bads153	NA	1	0.610789	0.778457	0.8434347	0.7380952	0.780720058	0.761905	0.87831	0.785714	0.761905	0.666667	0.547619	-0.275454
bax	NA	NA	1	0.783133	0.4787967	0.7065995	0.527696746	0.227549	0.564513	0.682647	0.526956	0.275454	0.922172	0.078313
bcl2	NA	NA	NA	1	0.6969825	0.9461247	0.773137093	0.275454	0.662689	0.970077	0.814386	0.491027	0.89822	-0.5
Caspase 3	NA	NA	NA	NA	1	0.6626987	0.728450578	0.747042	0.80253	0.698846	0.855484	0.747042	0.445815	-0.212125
Caspase 9	NA	NA	NA	NA	NA	1	0.634335047	0.214286	0.561143	0.97619	0.690476	0.357143	0.857143	-0.503003
IkappaBalpha	NA	NA	NA	NA	NA	NA	1	0.561143	0.9	0.731925	0.756323	0.902708	0.561143	-0.355889
fak	NA	NA	NA	NA	NA	NA	NA	1	0.731925	0.309524	0.452381	0.738095	0.02381	0.167668
jnk	NA	NA	NA	NA	NA	NA	NA	NA	1	0.609938	0.731925	0.853913	0.463553	-0.134992
lck	NA	NA	NA	NA	NA	NA	NA	NA	NA	1	0.738095	0.47619	0.833333	-0.538932
nfkb	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	1	0.619048	0.571429	-0.514979
p38	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	1	0.238095	-0.119763
pyk2	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	1	-0.227549
src	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	1

Task 1.2: Generate the phosphoactivation map of *Francisella* LVS Lipopolysaccharide (LPS) treated J774A.1 macrophage cells.

We were able to procure Francieslla LVS lipopolysaccharide from Biodefense and Emerging Infectious Disease Resources (Manassas, VA), which was of better quality that the LPS that we made. In order to confirm the quality of the LPS, we ran a gel (Figure 3) and confirmed that the LPS is of high quality.
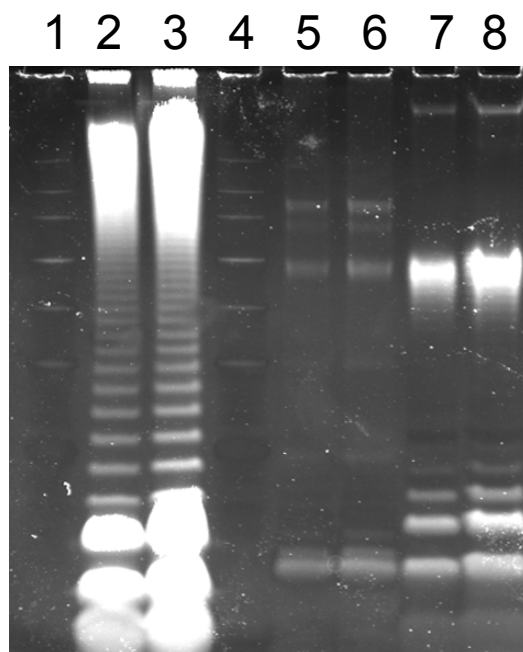
Figure 1.2A: Pro-Q Emerald 300 Lipopolysaccharide Stain of an SDS-PAGE gel. LPS from F. LVS, Pseudomonas and E.coli were run on a 12% SDS PAGE and was stained with Pro-Q Emerald 300 Lipopolysaccharide Stain Kit (Invitrogen, Cat. P20495). Lanes 1 and 4, Perfect Protein Markers (Novagen, Cat. No. 69079); lanes 2 and 3, F. LVS LPS, 25 and 50ug, respectively; lanes 5 and 6, Pseudomonas LPS, 30 and 60ug, respectively; Lanes 7 and 8, E. Coli LPS, 30 and 60ug, respectively.

Following this, we applied the 5ug/ml LPS was applied in 2ml/well media to the J774A.1 cells, and performed RPMA analysis of the resulting cellular lysates. The data obtained is presented below. These data will contribute to the phospho-protein mapping of murine cells in their response to factors from *Francisella* such as LPS. From the literature on J774A.1 cells, we expect to see no or relatively small effects on signaling as a result of LPS application (Hrstka et al, 2005 and Telepnev et al, 2003). This agrees with our findings (Figure 1.2), in which we examined the changes in many different molecules following LPS treatment of J774A.1.

Table 1.2:

J774A.1 cells (murine macrophages) were treated with Francisella LVS LPS (BEI Resources, Manassas, VA), 5ug/ml LPS was applied in 2ml/well media. Fold changes are given for the phosphorylation of the respective

	0	15	30	60	120	240	360
bads112	1.000	0.795	0.591	0.787	1.031	0.890	0.819
bads155	1.000	0.514	0.316	0.474	0.538	0.393	0.591
bax	1.000	0.358	0.377	0.531	0.500	0.388	0.577
bcl2	1.000	0.866	0.928	1.052	1.062	0.835	1.010
Caspase 3	1.000	0.978	0.511	0.661	1.089	1.017	1.117
Caspase 9	1.000	0.981	0.883	1.068	1.146	0.951	1.000
IkappaBalp	1.000	0.694	0.599	0.739	0.771	0.771	0.790
fak	1.000	0.958	0.844	1.125	1.229	1.073	1.063
jnk	1.000	0.439	0.684	0.337	0.571	0.408	0.622
lck	1.000	0.976	0.788	1.035	1.435	1.259	1.212
nfkb	1.000	1.075	1.061	0.823	0.932	0.816	0.728
p38	1.000	0.931	0.566	0.409	0.421	0.384	0.465
pyk2	1.000	0.760	0.493	1.027	1.100	0.780	1.020
src	1.000	0.447	0.412	0.508	0.822	0.298	0.511

antibody vs the time course of infection (minutes). Time zero represents the application of the substance, spinning the plate for 5 minutes to promote close contact with macrophage cells, and then removal, washing and lysis of sample.

Figure 1.2B: J774A.1 cells (murine macrophages) were treated with Francisella LVS LPS (BEI Resources, Manassas, VA), 5ug/ml LPS was applied in 2ml/well media. Fold changes are illustrated on the Y-axis in the phosphorylation of the respective antibody and the time course of infection (minutes) is on the X-axis. Time zero represents the application of the substance, spinning the plate for 5 minutes to promote close contact with macrophage cells, and then removal, washing and lysis of sample.

Relative Change in ppl'n with F. LVS LPS Rx of J774A.1 cells

- 19 -
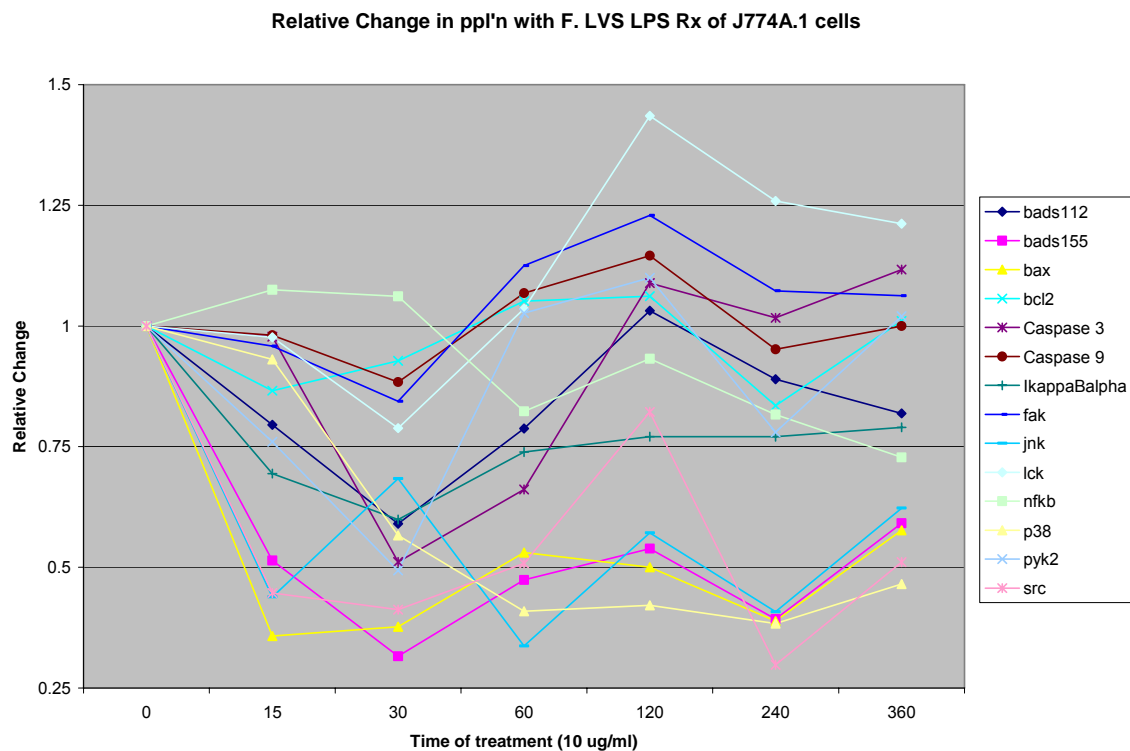
Conclusion: Francisella Live Vaccine Strain lipopolysaccharide (LPS) (Gunn and Ernst, 2007) was applied to J774A.1 murine macrophages for varying time. For several molecules (bcl2, bad S112, Caspase 9), very little change over time is seen with LPS treatment (~20% at early time and then return to normal.) For jnk, a strong down regulation was seen with LPS treatment. For Caspase 3, bax, and Bad S155, as well as p38, Ikba, NFKB, as well as src, strong change in activation was observed. Pyk2 can not be considered at this time, due to the problems validating this antibody. Fak and lck have not been validated at this time and so can also not be considered.

The strong change in activation of Src is corroborated by other studies showing that Francisella activation of macrophages requires SHIP (Parsa et al, 2007) and that in general, an LPS response by macrophages signals via SHIP (Fang et al, 2004). Furthermore, this activation pattern is different than the pattern observed following infection of J774A.1 cells by the entire organism Francisella LVS, as seen in Task 1.1.

Task 1.3: Compare the phosphoactivation profiles generated by RPMA to published results in J774A.1 cells and provide conclusions regarding the validation of RPMA technology.

See "Multiple Tasks 1.3, 1.5 and 1.8" section for a complete discussion of each molecule and how it changes.

Task 1.4: Generate the phosphoactivation map of *Francisella* LVS infected human activated THP-1 (macrophage) cells. Compare the phosphoactivation profiles between J774A.1 and THP-1 cells. Provide conclusions regarding the differences in response of human and mouse cells to *Francisella* LVS infection.

To confirm infection of THP-1 cells with *F. tularensis* LVS, we determined the CFUs of bacteria inside THP-1 cells after 22 hours of infection.

THP-1 Cells Infected with F.LVS

Figure 1.4A: THP-1 cells were split and treated with 100nM PMA for 48 hours. Cells were then pre-infected with 100MOI F.LVS, followed by incubating with 50ug/ml gentamicin for 1 hour, and 2ug/ml gentamicin for 22hours. Cells were lysed at different time points. Bacteria CFUs were determined by plating the cell lysate on chocolate agar plates.

To confirm that infection over the 4 hour time-course used in the RPMA analysis does not cause apoptosis of THP-1 cells, we performed an LDH-release assay, which measures cell death, and has been shown to be highly correlated to apoptosis.

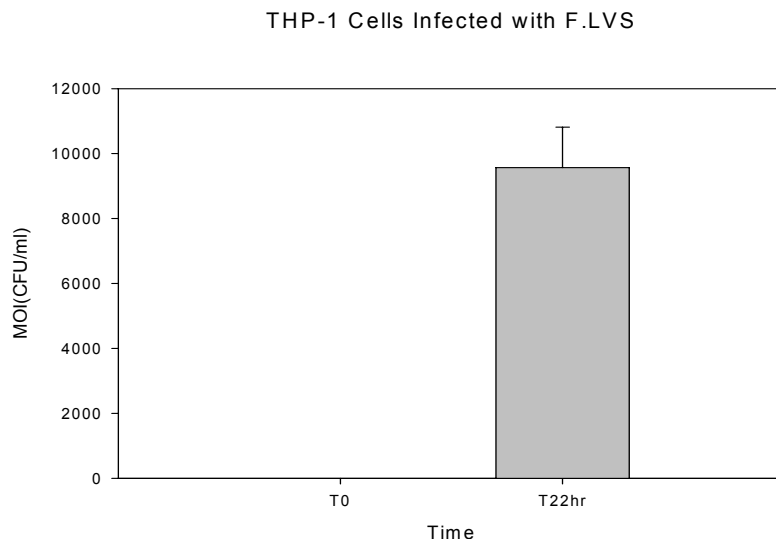## LDH Assay of THP-1 Cells Infected with F. LVS
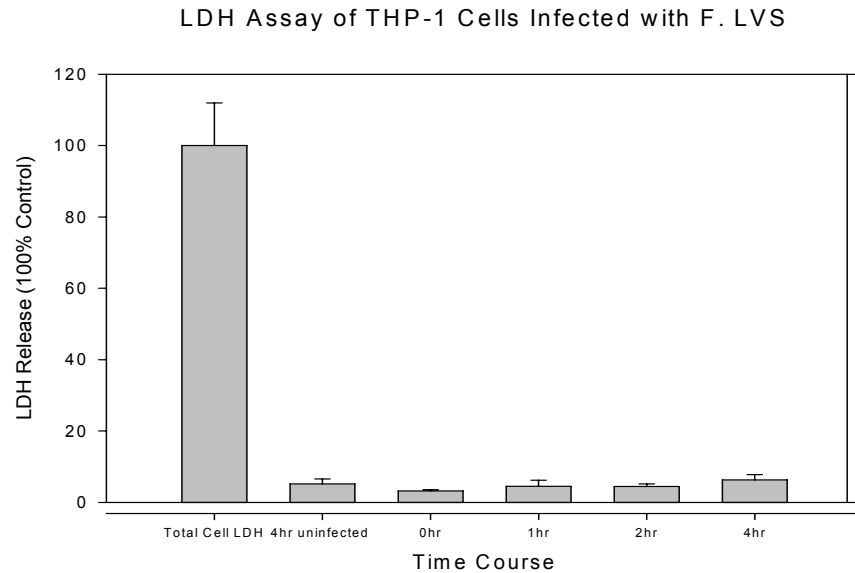
Figure 1.4B: THP-1 cells were split and treated with 100nM PMA for 48 hours. Cells were then pre-infected with 100MOI F.LVS, followed by incubating with 50ug/ml gentamicin for 1 hour, and 2ug/ml gentamicin for the indicated time. Promega CytoTox96 was used to determine the LDH Release, as a measure of apoptosis.

In Figure 1.4A & B we present data which confirm that THP-1 cells could be infected with *F. tularensis* LVS, and that infection over the 4 hour time-course used in the RPMA analysis does not cause apoptosis of THP-1 cells. We then examined *Francisella* LVS infected human activated THP-1 (macrophage) cells for the phosphorylation of different molecules (Figure 4.1C).

Figure 1.4C: THP-1 cells (human macrophages) were infected with *Francisella* LVS. Fold changes are illustrated on the Y-axis in the phosphorylation of NFKB, and the time course of infection (minutes) is on the X-axis. The graph represents the ratio of change between uninfected and infected THP-1 cells in this experiment.

Protein Modifications in F.LVS Infected THP-1 Cells
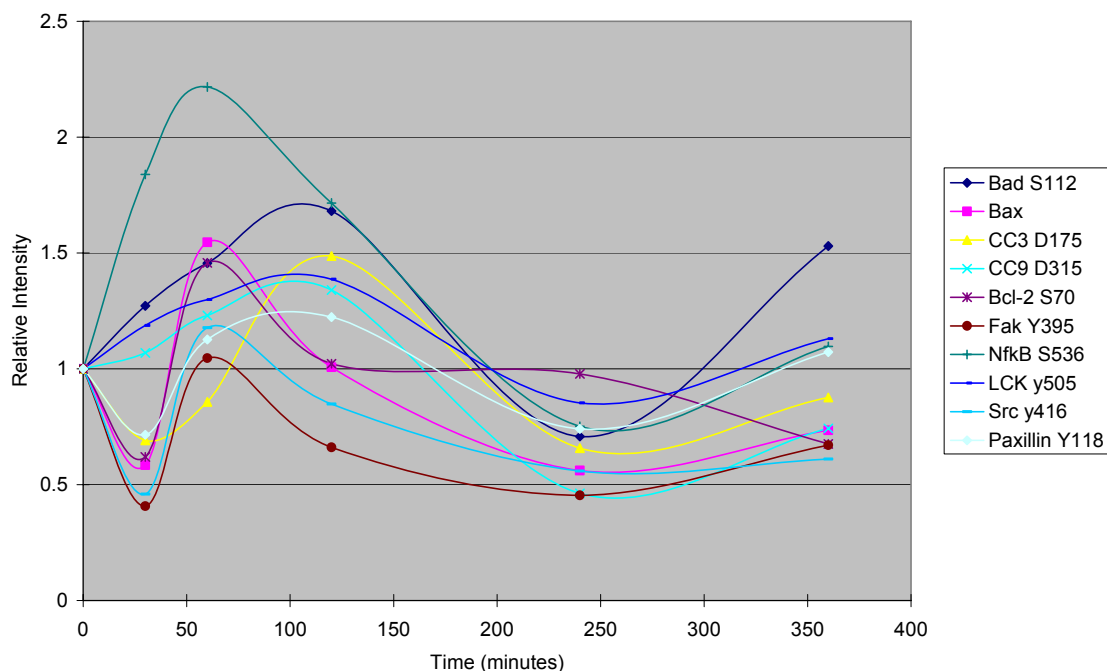
Table 1.4: This Table summarizes the data illustrated in Figure 1.4C above for easier reading of the changes.

time in minutes	0	30	60	120	240	360
bads112	1	1.27	1.46	1.68	0.71	1.53
bax	1	0.58	1.55	1.01	0.56	0.73
bcl2	1	0.62	1.46	1.02	0.98	0.68
Caspase 3	1	0.69	0.85	1.49	0.66	0.88
Caspase 9	1	1.07	1.23	1.34	0.46	0.74
fak	1	0.41	1.05	0.66	0.45	0.67
lck	1	1.19	1.3	1.39	0.85	1.13
nfkb	1	1.84	2.22	1.72	0.75	1.1
src	1	0.46	1.18	0.85	0.56	0.61
Paxillin	1	0.72	1.13	1.22	0.74	1.07

LVS Infected THP-1 Cells

Conclusion: Francisella LVS was found to be moderately activating for host cells. **NFKB**: When the relative changes were calculated, it was found that NFKB phosphorylation changes up to two-fold in infected vs. uninfected cells following *Francisella LVS* infection, with maximal changes observed around 60 minutes. This is a stronger activation than was observed for *F. novicida or F. tularensis* B38 infection of J774A.1 cells (See Figures 7B and 8B below), but correlates well with the levels of NFKB activation (phosphorylation) observed in J774A.1 cells infected with *F. tularensis* LVS. (See Task 1.1 above). **BAD S112**: Interestingly, we did not find a similar activation of pBAD (S112) phosphorylation when J774A.1 cells were infected with *F. tularensis* B38 (Task 1.5) or *F. novicida* (Task 1.7).

Task 1.5: Generate the phosphoactivation map of *Francisella tularensis B38* infected J774A.1 macrophage cells. Compare the phosphoactivation profiles generated to published results with other *Francisella* strains in J774A.1 cells for validation.

For this task, we infected that J774A.1 cells with *Francisella tularensis tularensis* NIHB38 bacteria (F. B38) for the times indicated, and we performed RPMA analysis for fourteen antibodies analyzed using RPMA. These data include a correction to a calculation which was incorrect in the Q4 quarterly report.

Figure 1.5A: Activation Profile of *Francisella tularensis* NIHB38 infected J774A.1 macrophages. This graph (Figure 1.5A) represents the RPMA data for both uninfected and infected cells at the various time of the experiment. Time zero was set to one for each data set for easy comparison. These data are also summarized in the Table 1.1A below.

F. tularensis NIH B38 RPMA
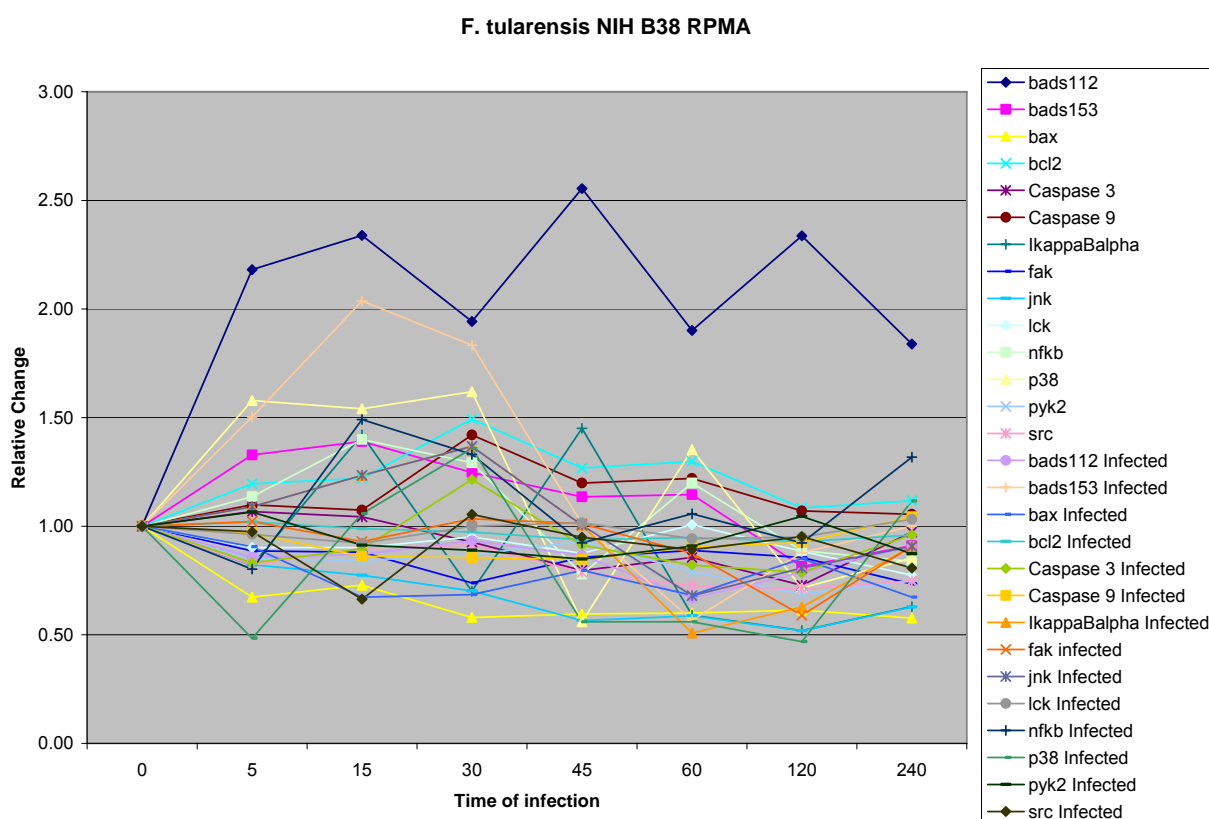
Figure 1.5B: Relative Changes in Activation of *Francisella tularensis* NIHB38 infected vs. uninfected J774A.1 macrophages. In this figure, the infected numbers were divided by the uninfected numbers to illustrate the fold change in signal for each antibody. This representation of fold-change simplifies the data set and allows up to observe which molecules are significantly up-regulated (greater than 1.5) or down-regulated (less than 0.5) following infection by Francisella B38.

F. tularensis tularensis B38 Relative Change by RPMA

Chart: Y-axis "Relative Change (infected/uninfected)" from 0 to 2.5; X-axis "Time of Infection" with values 0, 5, 15, 30, 45, 60, 120, 240. Legend: bads112, bads153, bax, bcl2, Caspase 3, Caspase 9, IkappaBalpha, fak, jnk, lck, nfkb, p38, pyk2, src.

Table 1.5A: The relative changes detected by RPMA are summarized in the Table below for Uninfected and B38 infected J774A.1 cells.

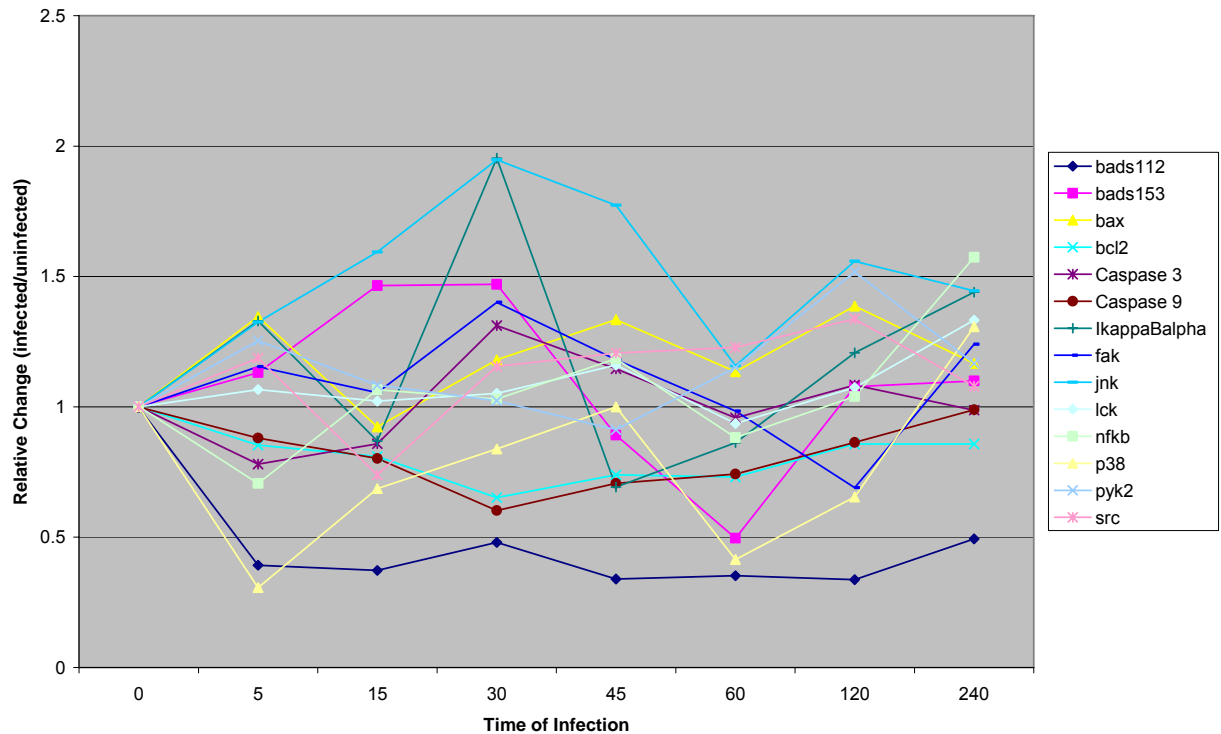|	Uninfected								B38 Infected							
time in minutes	0	5	15	30	45	60	120	240	0	5	15	30	45	60	120	240
bads112	1.00	2.18	2.34	1.94	2.55	1.90	2.34	1.84	1.00	0.86	0.87	0.93	0.87	0.67	0.79	0.91
bads153	1.00	1.33	1.39	1.25	1.13	1.14	0.82	0.91	1.00	1.50	2.04	1.83	1.01	0.57	0.88	1.00
bax	1.00	0.67	0.73	0.58	0.60	0.60	0.61	0.58	1.00	0.91	0.67	0.68	0.80	0.68	0.85	0.67
bcl2	1.00	1.19	1.22	1.49	1.27	1.30	1.08	1.12	1.00	1.02	0.99	0.97	0.94	0.95	0.93	0.96
Caspase 3	1.00	1.07	1.04	0.93	0.80	0.86	0.73	0.97	1.00	0.83	0.90	1.22	0.91	0.82	0.79	0.96
Caspase 9	1.00	1.10	1.07	1.42	1.20	1.22	1.07	1.06	1.00	0.97	0.86	0.85	0.85	0.90	0.92	1.04
IkappaBalpha	1.00	0.82	1.42	0.70	1.45	0.59	0.52	0.63	1.00	1.09	1.23	1.37	1.00	0.51	0.63	0.91
fak	1.00	0.89	0.88	0.74	0.86	0.89	0.86	0.73	1.00	1.02	0.93	1.03	1.01	0.87	0.59	0.91
jnk	1.00	0.82	0.77	0.70	0.57	0.59	0.52	0.63	1.00	1.09	1.23	1.37	1.00	0.68	0.81	0.91
lck	1.00	0.90	0.90	0.95	0.88	1.01	0.88	0.77	1.00	0.96	0.92	1.00	1.02	0.94	0.95	1.03
nfkb	1.00	1.14	1.40	1.29	0.78	1.20	0.89	0.84	1.00	0.80	1.49	1.33	0.92	1.06	0.92	1.32
p38	1.00	1.58	1.54	1.62	0.56	1.35	0.72	0.85	1.00	0.48	1.06	1.36	0.56	0.56	0.47	1.12
pyk2	1.00	0.85	0.84	0.87	0.93	0.79	0.69	0.75	1.00	1.07	0.91	0.89	0.85	0.91	1.05	0.87
src	1.00	0.82	0.90	0.91	0.79	0.73	0.71	0.75	1.00	0.97	0.66	1.05	0.95	0.89	0.95	0.81

Unsupervised clustering is done by the computer in the gereration of the heat-map illustrated. This approach allows the computer to group the data according to the samples that are most similar. This approach may reveal clustering of signalling pathways, as has been published for other experiments using RPMA (Espina et al, 2004, Liotta et al, 2003). In this case, different than the LVS infection heat map, there is not as much clustering of the apopotsis related molecules, Caspase 9, bcl-2, Bax, BadS153, Caspase 3, and BadS112 (the pivot point of IKBA and BadS112 can be rotated). Nor is the second cluster (includes IkBa, jnk, p38, Pyk2 (which has to be exluded, see validation above), src, and NFKB) strongly clusterd. This reflects

the more "quiet" effect that F. B38 has on host cells, not activating host molecules to such a high extent as F. LVS or F. novicida.

Figure 1.5C: Heat Map. This data represents unsupervised clustering of the infection data represented in Table 1.5A.
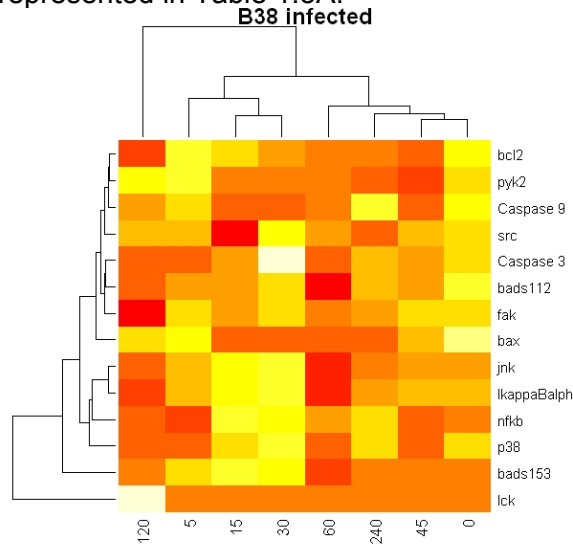

Table 1.5B: Spearman Rho Correlation calculation: In this table, the p-values of the SpearmanRho correlation calculation are presented. This analysis looks for molecules that have similar patterns of change over the timecourse of the experiment. Thus, molecules which have significant p-values are likely to change in the same general way in the experiment. This may indicate that these molecules are on the same pathway as each other.

P-value   Uninfected

	bads112	bads153	bax	bcl2	Caspase 3	Caspase 9	IkappaBalpha	fak	jnk	lck	nfkb	p38	pyk2	src
bads112	4.96E-05	0.582143	0.934871	0.5007937	0.46180556	0.3893849	0.461805556	0.6190972	0.216171	0.299206	0.840129	0.582143	0.840129	0.500794
bads153	NA	4.96E-05	0.619097	0.1511409	0.11498016	0.2674603	0.243055556	0.5007937	0.216171	0.326835	0.036756	0.036756	0.461806	0.243056
bax	NA	NA	4.96E-05	0.1511409	0.26746032	0.196627	0.359871032	0.0217758	0.170982	0.461806	0.619097	0.934871	0.500794	0.389385
bcl2	NA	NA	NA	4.96E-05	0.70332341	0.0022321	0.840128968	0.5364087	0.619097	0.461806	0.299206	0.299206	0.793006	0.976786
Caspase 3	NA	NA	NA	NA	4.96E-05	0.4618056	0.326835317	0.359871	0.004563	0.664583	0.26746	0.11498	0.500794	0.11498
Caspase 9	NA	NA	NA	NA	NA	4.96E-05	0.881994048	0.6645833	0.427877	0.461806	0.427877	0.359871	0.840129	0.793006
IkappaBalpha	NA	NA	NA	NA	NA	NA	4.96E-05	0.6190972	0.299206	0.976786	1	1	0.036756	0.096181
fak	NA	NA	NA	NA	NA	NA	NA	4.96E-05	0.196627	0.069395	0.500794	0.664583	0.359871	0.500794
jnk	NA	NA	NA	NA	NA	NA	NA	NA	4.96E-05	0.299206	0.26746	0.11498	0.170982	0.015377
lck	NA	NA	NA	NA	NA	NA	NA	NA	NA	4.96E-05	0.096181	0.151141	0.326835	0.326835
nfkb	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	4.96E-05	0.010714	0.976786	0.326835
p38	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	4.96E-05	0.703323	0.196627
pyk2	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	4.96E-05	0.021776
src	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	4.96E-05

.

P-value   B38

|	bads112	bads153	bax	bcl2	Caspase 3	Caspase 9	IkappaBalpha	fak	jnk	lck	nfkb	p38	pyk2	src
bads112	4.96E-05	0.299206	0.934871	0.299206	0.0011409	0.793006	0.170982143	0.196627	0.170982	0.752034	0.359871	0.045833	0.427877	0.326835
bads153	NA	4.96E-05	0.582143	0.170982	0.2674603	0.2992063	0.001140873	0.057589	0.001141	0.326835	0.389385	0.26746	0.752034	0.840129
bax	NA	NA	4.96E-05	0.619097	0.840129	0.5821429	0.881994048	0.619097	0.881994	0.664583	0.027927	0.132292	0.11498	0.069395
bcl2	NA	NA	NA	4.96E-05	0.359871	0.4618056	0.114980159	0.151141	0.11498	0.151141	0.840129	0.500794	0.326835	0.500794
Caspase 3	NA	NA	NA	NA	4.96E-05	8.82E-01	0.151140873	0.096181	0.151141	0.881994	0.299206	0.015377	0.216171	0.299206
Caspase 9	NA	NA	NA	NA	NA	4.96E-05	0.326835317	0.389385	0.326835	0.536409	0.582143	0.793006	0.326835	0.793006
IkappaBalpha	NA	NA	NA	NA	NA	NA	4.96E-05	0.015377	4.96E-05	0.389385	0.427877	0.216171	0.881994	0.389385
fak	NA	NA	NA	NA	NA	NA	NA	4.96E-05	0.015377	0.582143	0.934871	0.389385	0.752034	0.096181
jnk	NA	NA	NA	NA	NA	NA	NA	NA	4.96E-05	0.389385	0.427877	0.216171	0.881994	0.389385
lck	NA	NA	NA	NA	NA	NA	NA	NA	NA	4.96E-05	0.389385	0.703323	0.664583	0.664583
nfkb	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	4.96E-05	0.015377	0.26746	0.389385
p38	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	4.96E-05	0.132292	0.976786
pyk2	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	4.96E-05	0.582143
src	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	4.96E-05

Table 1.5C: Spearman Rho Correlation calculation: In this table, the Rho correlation of the SpearmanRho correlation calculation are presented. All pairs with significant P-values in the Table above have significant Rho correlations in the Table below.

Uninfected
Rho (estimate of correlation)

	bads112	bads153	bax	bcl2	Caspase 3	Caspase 9	IkappaBalpha	fak	jnk	lck	nfkb	p38	pyk2	src
bads112	1	0.227549	0.036364	0.275454	-0.3113828	0.3493976	0.311382828	-0.212125	-0.49103	-0.412129	-0.083834	-0.239525	-0.083834	-0.275454
bads153	NA	1	0.204834	0.5714286	0.61904762	0.4431217	0.476190476	0.2771285	0.5	0.409668	0.761905	0.761905	0.309524	0.47619
bax	NA	NA	1	-0.5542571	0.45786456	-0.5212217	0.373521087	0.804878	0.530159	0.29878	0.216883	0.036147	0.289178	0.361472
bcl2	NA	NA	NA	1	-0.1666667	0.9221722	0.095238095	-0.25303	-0.21429	0.301227	0.428571	0.428571	0.119048	0.02381
Caspase 3	NA	NA	NA	NA	1	-0.3113828	0.404761905	0.3855702	0.904762	0.192785	0.452381	0.619048	0.285714	0.619048
Caspase 9	NA	NA	NA	NA	NA	1	-0.071857576	-0.175761	-0.32336	0.315157	0.323359	0.38324	0.083834	-0.107786
IkappaBalpha	NA	NA	NA	NA	NA	NA	1	0.2168832	0.428571	-0.012049	0	0	0.761905	0.642857
fak	NA	NA	NA	NA	NA	NA	NA	1	0.51811	0.689024	0.277129	0.180736	0.38557	0.289178
jnk	NA	NA	NA	NA	NA	NA	NA	NA	1	0.433766	0.452381	0.619048	0.547619	0.833333
lck	NA	NA	NA	NA	NA	NA	NA	NA	NA	1	0.638601	0.554257	0.397619	0.409668
nfkb	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	1	0.857143	0.02381	0.404762
p38	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	1	0.166667	0.52381
pyk2	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	1	0.809524
src	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	1

B38
Rho (estimate of correlation)

	bads112	bads153	bax	bcl2	Caspase 3	Caspase 9	IkappaBalpha	fak	jnk	lck	nfkb	p38	pyk2	src
bads112	1	0.421687	0.030304	0.431145	0.9461247	0.1144578	0.548192771	0.526956	0.548193	0.144578	0.36747	0.728916	-0.331325	0.403614
bads153	NA	1	-0.236368	0.538932	0.4431217	-0.421687	0.957831325	0.718576	0.957831	-0.391566	0.349398	0.457831	-0.13253	0.096386
bax	NA	NA	1	0.216883	-0.0963925	0.2242465	-0.072728608	0.204834	-0.072729	0.187882	-0.787893	-0.600011	0.606072	0.690922
bcl2	NA	NA	NA	1	0.3809524	0.2994066	0.610789394	0.571429	0.610789	-0.574861	0.083834	0.275454	0.395217	0.275454
Caspase 3	NA	NA	NA	NA	1	-5.99E-02	0.574860606	0.642857	0.574861	0.059881	0.431145	0.826362	-0.503003	0.431145
Caspase 9	NA	NA	NA	NA	NA	1	-0.403614458	-0.359288	-0.403614	0.246988	-0.222892	-0.114458	0.39759	-0.114458
IkappaBalpha	NA	NA	NA	NA	NA	NA	1	0.826362	1.00E+00	-0.355422	0.319277	0.5	-0.060241	0.355422
fak	NA	NA	NA	NA	NA	NA	NA	1	0.826362	-0.227549	-0.035929	0.359288	-0.143715	0.634742
jnk	NA	NA	NA	NA	NA	NA	NA	NA	1	-0.355422	0.319277	0.5	-0.060241	0.355422
lck	NA	NA	NA	NA	NA	NA	NA	NA	NA	1	-0.349398	-0.156627	-0.192771	0.180723
nfkb	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	1	0.837349	-0.445783	-0.349398
p38	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	1	-0.596386	-0.006024
pyk2	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	1	0.240964
src	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	1

Figure 1.5.D: **Validation of RPMA data**: Phosphorylation of NFKB across a *F. tularensis tularensis* B38 infection time course was validated by Western Blotting lysates following larger scale infection of J774A.1 cells for the time indicated. 0U= zero uninfected, 4U= four hour uninfected. T0 = time zero infected, in which the cells have had bacteria applied, spun down in

the centrifuge, gentamycin applied and then washed and lysed. These data confirm that the level of phospho NFKB change over the time course of infection, while the amount of total NFKB does not change.
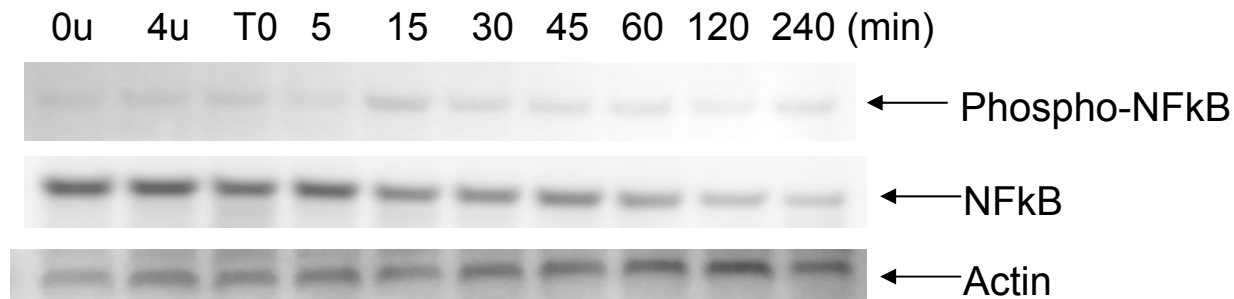
0u   4u   T0   5   15   30   45   60   120   240 (min)

← Phospho-NFkB

← NFkB

← Actin

Conclusion: Following infection by NIHB38 strain of Francisella, the host J774A.1 cells are less activated overall than infection by Francisella novicida strain. We noted that molecules of the TLR pathway are activated following B38 infection, including Jnk, IKBa and NFKB. We also noted that BasS112, p38 and BadS153 were down regulated.

Task 1.6: Analyze and compare the phosphoactivation profiles generated by the type strain B38 to vaccine (LVS) strain. Provide conclusions regarding the differences between the strains of *Francisella*.

Table 1.6A: The relative changes detected by RPMA are summarized in the Table below for LVS and B38 infected J774A.1 cells.

LVS Infected								B38 Infected							
0	5	15	30	45	60	120	240	0	5	15	30	45	60	120	240
1.00	1.18	1.54	1.49	1.27	1.21	1.36	0.90	1.00	0.86	0.87	0.93	0.87	0.67	0.79	0.91
1.00	1.19	1.36	1.23	1.12	1.18	1.21	0.80	1.00	1.50	2.04	1.83	1.01	0.57	0.88	1.00
1.00	1.04	1.16	1.11	1.09	1.04	1.21	1.10	1.00	0.91	0.67	0.68	0.80	0.68	0.85	0.67
1.00	1.12	1.36	1.16	1.18	1.12	1.22	1.07	1.00	1.02	0.99	0.97	0.94	0.95	0.93	0.96
1.00	1.18	1.20	1.11	1.00	0.93	1.18	0.85	1.00	0.83	0.90	1.22	0.91	0.82	0.79	0.96
1.00	1.18	1.32	1.21	1.28	1.20	1.37	0.94	1.00	0.97	0.86	0.85	0.85	0.90	0.92	1.04
1.00	1.29	1.64	1.63	1.35	1.00	1.17	1.00	1.00	1.09	1.23	1.37	1.00	0.51	0.63	0.91
1.00	1.07	1.34	1.11	0.87	0.93	0.96	0.90	1.00	1.02	0.93	1.03	1.01	0.87	0.59	0.91
1.00	1.29	1.30	1.63	1.08	1.00	1.17	1.00	1.00	1.09	1.23	1.37	1.00	0.68	0.81	0.91
1.00	1.14	1.34	1.20	1.28	1.15	1.30	0.87	1.00	0.96	0.92	1.00	1.02	0.94	0.95	1.03
1.00	2.61	3.52	2.30	2.53	1.67	2.56	1.27	1.00	0.80	1.49	1.33	0.92	1.06	0.92	1.32
1.00	1.31	1.55	1.38	1.02	0.68	0.95	0.73	1.00	0.48	1.06	1.36	0.56	0.56	0.47	1.12
1.00	1.08	1.47	1.37	1.41	1.24	1.74	1.36	1.00	1.07	0.91	0.89	0.85	0.91	1.05	0.87
1.00	0.90	0.90	0.99	0.68	0.83	0.93	1.14	1.00	0.97	0.66	1.05	0.95	0.89	0.95	0.81

Table 1.6B: Spearman Rho Correlation calculation: In this table, the p-values of the SpearmanRho correlation calculation are presented. This analysis looks for molecules that have similar patterns of change over the time course of the experiment. Thus, molecules which have significant p-values are likely to change in the same general way in the experiment. This may indicate that these molecules are on the same pathway as each other.

P-value Uninfected

	bads112	bads153	bax	bcl2	Caspase 3	Caspase 9	IkappaBalpha	fak	jnk	lck	nfkb	p38	pyk2	src
bads112	4.96E-05	0.582143	0.934871	0.5007937	0.46180556	0.3893849	0.461805556	0.6190972	0.216171	0.299206	0.840129	0.582143	0.840129	0.500794
bads153	NA	4.96E-05	0.619097	0.1511409	0.11498016	0.2674603	0.243055556	0.5007937	0.216171	0.326835	0.036756	0.036756	0.461806	0.243056
bax	NA	NA	4.96E-05	0.1511409	0.26746032	0.196627	0.359871032	0.0217758	0.170982	0.461806	0.619097	0.934871	0.500794	0.389385
bcl2	NA	NA	NA	4.96E-05	0.70332341	0.0022321	0.840128968	0.5364087	0.619097	0.461806	0.299206	0.299206	0.793006	0.976786
Caspase 3	NA	NA	NA	NA	4.96E-05	0.4618056	0.326835317	0.359871	0.004563	0.664583	0.26746	0.11498	0.500794	0.11498
Caspase 9	NA	NA	NA	NA	NA	4.96E-05	0.881994048	0.6645833	0.427877	0.461806	0.427877	0.359871	0.840129	0.793006
IkappaBalpha	NA	NA	NA	NA	NA	NA	4.96E-05	0.6190972	0.299206	0.976786	1	1	0.036756	0.096181
fak	NA	NA	NA	NA	NA	NA	NA	4.96E-05	0.196627	0.069395	0.500794	0.664583	0.359871	0.500794
jnk	NA	NA	NA	NA	NA	NA	NA	NA	4.96E-05	0.299206	0.26746	0.11498	0.170982	0.015377
lck	NA	NA	NA	NA	NA	NA	NA	NA	NA	4.96E-05	0.096181	0.151141	0.326835	0.326835
nfkb	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	4.96E-05	0.010714	0.976786	0.326835
p38	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	4.96E-05	0.703323	0.196627
pyk2	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	4.96E-05	0.021776
src	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	4.96E-05

P-value F LVS

	bads112	bads153	bax	bcl2	Caspase 3	Caspase 9	IkappaBalpha	fak	jnk	lck	nfkb	p38	pyk2	src
bads112	4.96E-05	0.004563	0.069395	0.004563	0.0575893	0.0072421	0.015376984	0.196627	0.027927	0.002232	0.083085	0.11498	0.045833	0.326835
bads153	NA	4.96E-05	0.11498	0.027927	0.0107143	0.0458333	0.027926587	0.036756	0.007242	0.027927	0.036756	0.083085	0.170982	0.500794
bax	NA	NA	4.96E-05	0.027927	0.2430556	0.0575893	0.196626984	0.582143	0.151141	0.069395	0.196627	0.500794	0.002232	0.840129
bcl2	NA	NA	NA	4.96E-05	0.0575893	0.0011409	0.027926587	0.500794	0.083085	0.000397	0.021776	0.216171	0.004563	0.216171
Caspase 3	NA	NA	NA	NA	4.96E-05	0.0830853	0.045833333	0.036756	0.021776	0.057589	0.010714	0.036756	0.26746	0.619097
Caspase 9	NA	NA	NA	NA	NA	4.96E-05	0.096180556	0.619097	0.151141	0.000397	0.069395	0.389385	0.010714	0.216171
IkappaBalpha	NA	NA	NA	NA	NA	NA	4.96E-05	0.151141	0.004563	0.045833	0.036756	0.004563	0.151141	0.389385
fak	NA	NA	NA	NA	NA	NA	NA	4.96E-05	0.045833	0.461806	0.26746	0.045833	0.976786	0.703323
jnk	NA	NA	NA	NA	NA	NA	NA	NA	4.96E-05	0.11498	0.045833	0.010714	0.243056	0.752034
lck	NA	NA	NA	NA	NA	NA	NA	NA	NA	4.96E-05	0.045833	0.243056	0.015377	0.170982
nfkb	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	4.96E-05	0.11498	0.151141	0.196627
p38	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	4.96E-05	0.582143	0.793006
pyk2	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	4.96E-05	0.582143
src	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	4.96E-05

P-value B38

	bads112	bads153	bax	bcl2	Caspase 3	Caspase 9	IkappaBalpha	fak	jnk	lck	nfkb	p38	pyk2	src
bads112	4.96E-05	0.299206	0.934871	0.299206	0.0011409	0.793006	0.170982143	0.196627	0.170982	0.752034	0.359871	0.045833	0.427877	0.326835
bads153	NA	4.96E-05	0.582143	0.170982	0.2674603	0.2992063	0.001140873	0.057589	0.001141	0.326835	0.389385	0.26746	0.752034	0.840129
bax	NA	NA	4.96E-05	0.619097	0.840129	0.5821429	0.881994048	0.619097	0.881994	0.664583	0.027927	0.132292	0.11498	0.069395
bcl2	NA	NA	NA	4.96E-05	0.359871	0.4618056	0.114980159	0.151141	0.11498	0.151141	0.840129	0.500794	0.326835	0.500794
Caspase 3	NA	NA	NA	NA	4.96E-05	8.82E-01	0.151140873	0.096181	0.151141	0.881994	0.299206	0.015377	0.216171	0.299206
Caspase 9	NA	NA	NA	NA	NA	4.96E-05	0.326835317	0.389385	0.326835	0.536409	0.582143	0.793006	0.326835	0.793006
IkappaBalpha	NA	NA	NA	NA	NA	NA	4.96E-05	0.015377	4.96E-05	0.389385	0.427877	0.216171	0.881994	0.389385
fak	NA	NA	NA	NA	NA	NA	NA	4.96E-05	0.015377	0.582143	0.934871	0.389385	0.752034	0.096181
jnk	NA	NA	NA	NA	NA	NA	NA	NA	4.96E-05	0.389385	0.427877	0.216171	0.881994	0.389385
lck	NA	NA	NA	NA	NA	NA	NA	NA	NA	4.96E-05	0.389385	0.703323	0.664583	0.664583
nfkb	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	4.96E-05	0.015377	0.26746	0.389385
p38	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	4.96E-05	0.132292	0.976786
pyk2	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	4.96E-05	0.582143
src	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	4.96E-05

Table 1.6C: Spearman Rho Correlation calculation: In this table, the Rho correlation of the SpearmanRho correlation calculation are presented. All pairs with significant P-values in the Table above have significant Rho correlations in the Table below.

Uninfected
Rho (estimate of correlation)

	bads112	bads153	bax	bcl2	Caspase 3	Caspase 9	IkappaBalpha	fak	jnk	lck	nfkb	p38	pyk2	src
bads112	1	0.227549	0.036364	0.275454	-0.3113828	0.3493976	0.311382828	-0.212125	-0.49103	-0.412129	-0.083834	-0.239525	-0.083834	-0.275454
bads153	NA	1	0.204834	0.5714286	0.61904762	0.4431217	0.476190476	0.2771285	0.5	0.409668	0.761905	0.761905	0.309524	0.47619
bax	NA	NA	1	-0.5542571	0.45786456	-0.5212217	0.373521087	0.804878	0.530159	0.29878	0.216883	0.036147	0.289178	0.361472
bcl2	NA	NA	NA	1	-0.1666667	0.9221722	0.095238095	-0.25303	-0.21429	0.301227	0.428571	0.428571	0.119048	0.02381
Caspase 3	NA	NA	NA	NA	1	-0.3113828	0.404761905	0.3855702	0.904762	0.192785	0.452381	0.619048	0.285714	0.619048
Caspase 9	NA	NA	NA	NA	NA	1	-0.071857576	-0.175761	-0.32336	0.315157	0.323359	0.38324	0.083834	-0.107786
IkappaBalpha	NA	NA	NA	NA	NA	NA	1	0.2168832	0.428571	-0.012049	0	0	0.761905	0.642857
fak	NA	NA	NA	NA	NA	NA	NA	1	0.51811	0.689024	0.277129	0.180736	0.38557	0.289178
jnk	NA	NA	NA	NA	NA	NA	NA	NA	1	0.433766	0.452381	0.619048	0.547619	0.833333
lck	NA	NA	NA	NA	NA	NA	NA	NA	NA	1	0.638601	0.554257	0.397619	0.409668
nfkb	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	1	0.857143	0.02381	0.404762
p38	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	1	0.166667	0.52381
pyk2	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	1	0.809524
src	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	1

B38
Rho (estimate of correlation)

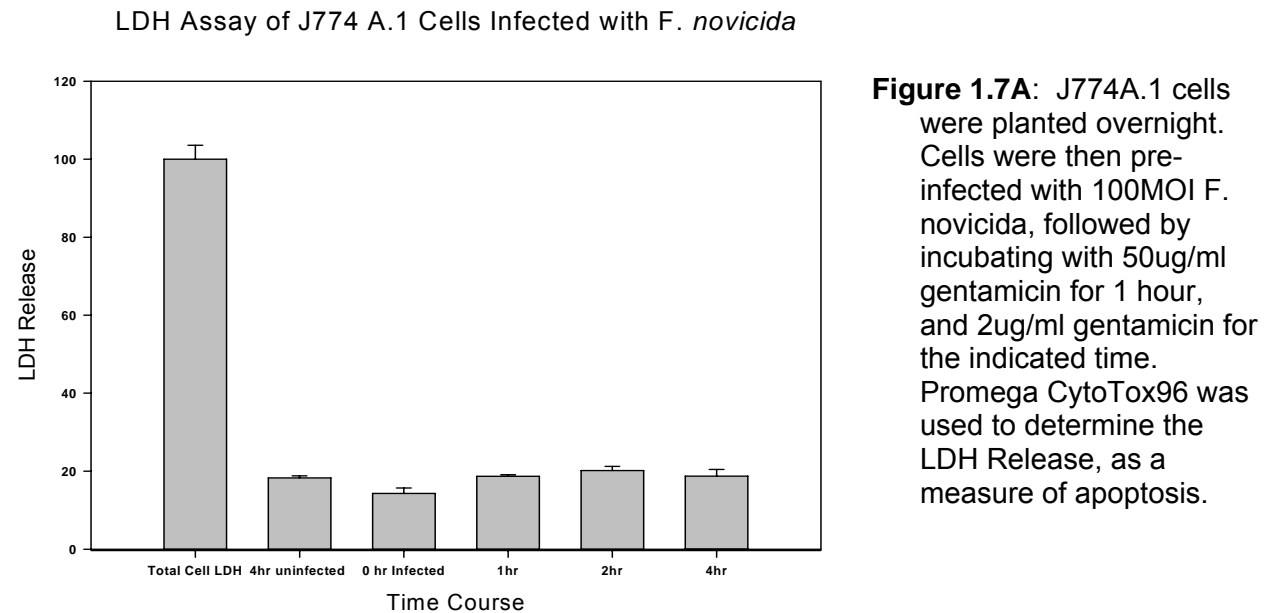|	bads112	bads153	bax	bcl2	Caspase 3	Caspase 9	IkappaBalpha	fak	jnk	lck	nfkb	p38	pyk2	src
bads112	1	0.421687	0.030304	0.431145	0.9461247	0.1144578	0.548192771	0.526956	0.548193	0.144578	0.36747	0.728916	-0.331325	0.403614
bads153	NA	1	-0.236368	0.538932	0.4431217	-0.421687	0.957831325	0.718576	0.957831	-0.391566	0.349398	0.457831	-0.13253	0.096386
bax	NA	NA	1	0.216883	-0.0963925	0.2242465	-0.072728608	0.204834	-0.072729	0.187882	-0.787893	-0.600011	0.606072	0.690922
bcl2	NA	NA	NA	1	0.3809524	0.2994066	0.610789394	0.571429	0.610789	-0.574861	0.083834	0.275454	0.395217	0.275454
Caspase 3	NA	NA	NA	NA	1	-5.99E-02	0.574860606	0.642857	0.574861	0.059881	0.431145	0.826362	-0.503003	0.431145
Caspase 9	NA	NA	NA	NA	NA	1	-0.403614458	-0.359288	-0.403614	0.246988	-0.222892	-0.114458	0.39759	-0.114458
IkappaBalpha	NA	NA	NA	NA	NA	NA	1	0.826362	1.00E+00	-0.355422	0.319277	0.5	-0.060241	0.355422
fak	NA	NA	NA	NA	NA	NA	NA	1	0.826362	-0.227549	-0.035929	0.359288	-0.143715	0.634742
jnk	NA	NA	NA	NA	NA	NA	NA	NA	1	-0.355422	0.319277	0.5	-0.060241	0.355422
lck	NA	NA	NA	NA	NA	NA	NA	NA	NA	1	-0.349398	-0.156627	-0.192771	0.180723
nfkb	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	1	0.837349	-0.445783	-0.349398
p38	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	1	-0.596386	-0.006024
pyk2	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	1	0.240964
src	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	1

F LVS
Rho (estimate of correlation)

	bads112	bads153	bax	bcl2	Caspase 3	Caspase 9	IkappaBalpha	fak	jnk	lck	nfkb	p38	pyk2	src
bads112	1	0.904762	0.682647	0.89822	0.6988459	0.8809524	0.829515062	0.52381	0.78072	0.928571	0.666667	0.619048	0.738095	-0.395217
bads153	NA	1	0.610789	0.778457	0.8434347	0.7380952	0.780720058	0.761905	0.87831	0.785714	0.761905	0.666667	0.547619	-0.275454
bax	NA	NA	1	0.783133	0.4787967	0.7065995	0.527696746	0.227549	0.564513	0.682647	0.526956	0.275454	0.922172	0.078313
bcl2	NA	NA	NA	1	0.6969825	0.9461247	0.773137093	0.275454	0.662689	0.970077	0.814386	0.491027	0.89822	-0.5
Caspase 3	NA	NA	NA	NA	1	0.6626987	0.728450578	0.747042	0.80253	0.698846	0.855484	0.747042	0.445815	-0.212125
Caspase 9	NA	NA	NA	NA	NA	1	0.634335047	0.214286	0.561143	0.97619	0.690476	0.357143	0.857143	-0.503003
IkappaBalpha	NA	NA	NA	NA	NA	NA	1	0.561143	0.9	0.731925	0.756323	0.902708	0.561143	-0.355889
fak	NA	NA	NA	NA	NA	NA	NA	1	0.731925	0.309524	0.452381	0.738095	0.02381	0.167668
jnk	NA	NA	NA	NA	NA	NA	NA	NA	1	0.609938	0.731925	0.853913	0.463553	-0.134992
lck	NA	NA	NA	NA	NA	NA	NA	NA	NA	1	0.738095	0.47619	0.833333	-0.538932
nfkb	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	1	0.619048	0.571429	-0.514979
p38	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	1	0.238095	-0.119763
pyk2	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	1	-0.227549
src	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	1

Conclusion: Following infection by NIHB38 strain of Francisella, the host J774A.1 cells are less activated overall than infection by Francisella LVS strain. We noted that while there are many Spearman correlations which are significant for the LVS strain, there are as few for NIHB38 as there are for the uninfected cells, indicating a more "quiet" host cell following infection.

Task 1.7: Generate the phosphoactivation map of *Francisella tularensis novicida* infected J774A.1 macrophage cells. Compare the phosphoactivation profiles generated to published results with other *Francisella* strains in J774A.1 cells for validation.

In the first experiment, we checked that short time course of infection by *F. novicida* does not cause cell death, as measured by the release of the intracellular enzyme Lactate Dehydrogenase (LDH). As described previously, this assay is highly correlated to measures of apoptosis. Thus, infected cells during the time course of the RPMA assay are not undergoing apoptosis.

LDH Assay of J774 A.1 Cells Infected with F. *novicida*

Figure 1.7A: J774A.1 cells were planted overnight. Cells were then pre-infected with 100MOI F. novicida, followed by incubating with 50ug/ml gentamicin for 1 hour, and 2ug/ml gentamicin for the indicated time. Promega CytoTox96 was used to determine the LDH Release, as a measure of apoptosis.

We infected J774A.1 cells with *Francisella novicida* bacteria for the times indicated, and we performed RPMA analysis. We present the data analysis of fourteen of the antibodies analyzed using RPMA.

Figure 1.7B: Activation profile of *Francisella novicida* infected J774A.1 macrophages.

Activation profile of F. novicida infected J774A.1 macrophages
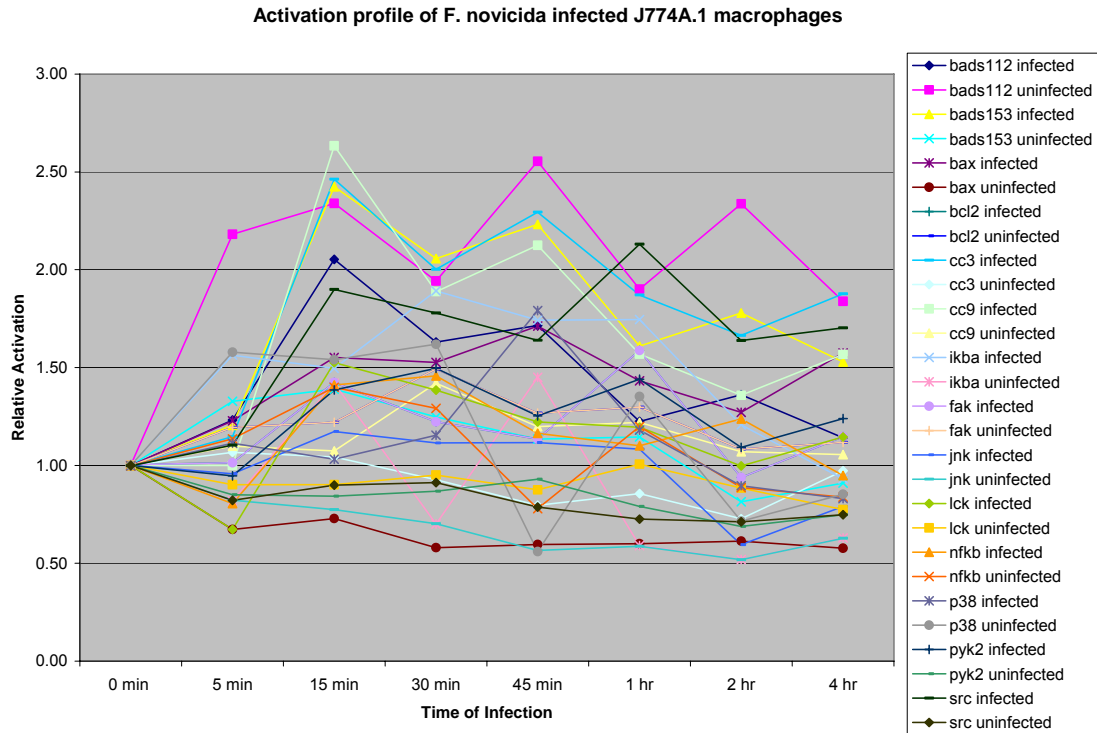
This graph (Figure 1.7A) represents the RPMA data for both uninfected and infected cells at the various time of the experiment. Time zero was set to one for each data set for easy comparison. These data are also summarized in the Table 1.7A below.

Figure 1.7C: Relative Changes in Activation of *F. novicida* infected vs. uninfected J774A.1 macrophages.

In this figure, the infected numbers were divided by the uninfected numbers to illustrate the fold change in signal for each antibody. This representation of fold-change simplifies the data set and allows up to observe which molecules are significantly up-regulated (greater than 1.5) or down-regulated (less than 0.5) following infection by *F. novicida*.
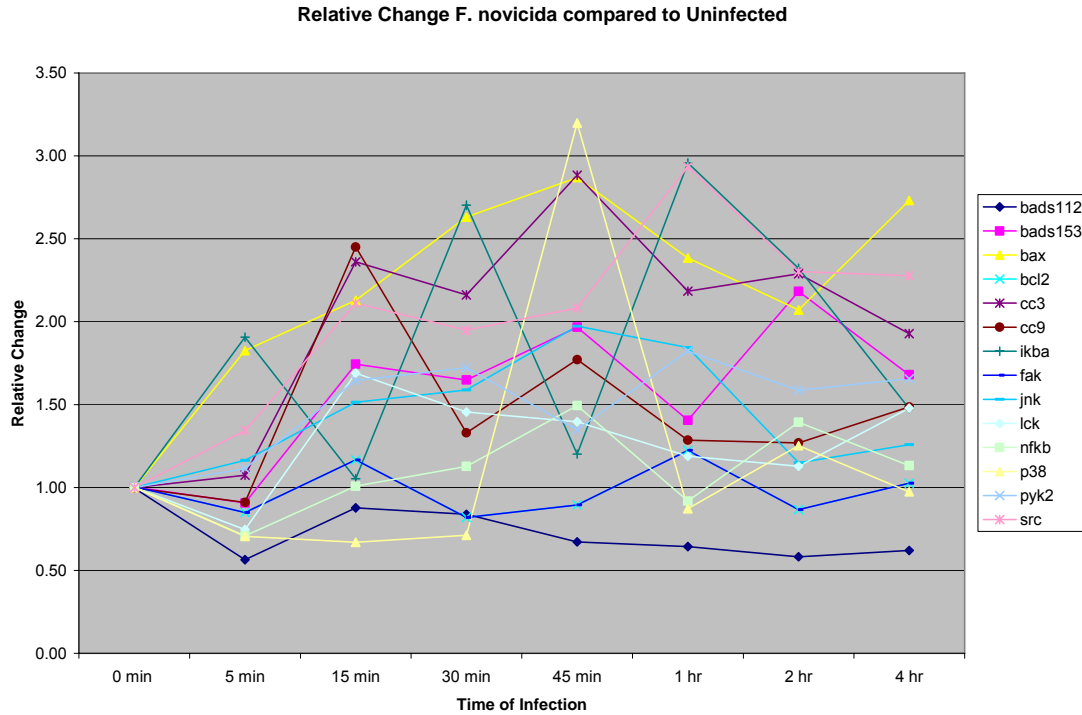
From this comparison in which we compared the uninfected levels to the *F. novicida* levels, we can see that significant changes are occurring in the activation of most of the molecules studied, with perhaps the exception of FAK and pBAD (S112). These findings are being confirmed by Western Blotting and will also be repeated to verify. *F. novicida* is the most "activating" of all the strains.

Table 1.7A: The relative changes detected by RPMA are summarized in the Table below for Uninfected and *F. novicida* infected J774A.1 cells.

	Uninfected								Novicida Infected							
time in minutes	0	5	15	30	45	60	120	240	0	5	15	30	45	60	120	240
bads112	1.00	2.18	2.34	1.94	2.55	1.90	2.34	1.84	1.00	1.23	2.05	1.63	1.72	1.22	1.36	1.14
bads153	1.00	1.33	1.39	1.25	1.13	1.14	0.82	0.91	1.00	1.20	2.42	2.06	2.23	1.61	1.78	1.53
bax	1.00	0.67	0.73	0.58	0.60	0.60	0.61	0.58	1.00	1.23	1.55	1.53	1.71	1.43	1.27	1.58
bcl2	1.00	1.19	1.22	1.49	1.27	1.30	1.08	1.12	1.00	1.01	1.42	1.22	1.14	1.59	0.94	1.15
Caspase 3	1.00	1.07	1.04	0.93	0.80	0.86	0.73	0.97	1.00	1.15	2.46	2.00	2.29	1.87	1.66	1.88
Caspase 9	1.00	1.10	1.07	1.42	1.20	1.22	1.07	1.06	1.00	1.00	2.63	1.89	2.12	1.57	1.36	1.57
IkappaBalpha	1.00	0.82	1.42	0.70	1.45	0.59	0.52	0.63	1.00	1.56	1.50	1.89	1.74	1.75	1.21	0.93
fak	1.00	0.89	0.88	0.74	0.86	0.89	0.86	0.73	1.00	1.52	2.71	2.02	2.13	3.02	1.43	2.68
jnk	1.00	0.82	0.77	0.70	0.57	0.59	0.52	0.63	1.00	0.96	1.17	1.12	1.12	1.08	0.60	0.79
lck	1.00	0.90	0.90	0.95	0.88	1.01	0.88	0.77	1.00	0.67	1.52	1.38	1.22	1.20	1.00	1.15
nfkb	1.00	1.14	1.40	1.29	0.78	1.20	0.89	0.84	1.00	0.81	1.41	1.46	1.16	1.10	1.24	0.95
p38	1.00	1.58	1.54	1.62	0.56	1.35	0.72	0.85	1.00	1.11	1.03	1.15	1.79	1.18	0.90	0.83
pyk2	1.00	0.85	0.84	0.87	0.93	0.79	0.69	0.75	1.00	0.95	1.39	1.50	1.25	1.44	1.09	1.24
src	1.00	0.82	0.90	0.91	0.79	0.73	0.71	0.75	1.00	1.10	1.90	1.78	1.64	2.13	1.64	1.70

Figure 1.7D: Heat Map. This data set represents unsupervised clustering of the LVS infection data represented in Table 1.1A. This approach allows the computer to group the data according to the samples that are most similar. This approach may reveal clustering of signalling pathways, as has been published for other experiments using RPMA (Espina et al,
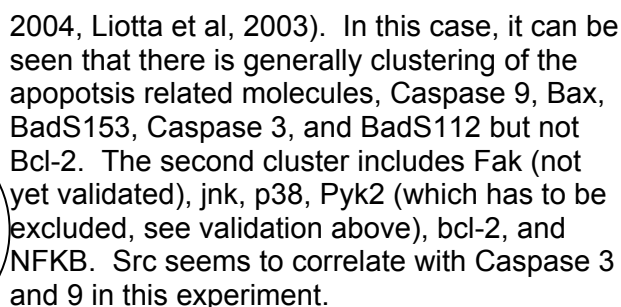
Novicida infected

2004, Liotta et al, 2003). In this case, it can be seen that there is generally clustering of the apopotsis related molecules, Caspase 9, Bax, BadS153, Caspase 3, and BadS112 but not Bcl-2. The second cluster includes Fak (not yet validated), jnk, p38, Pyk2 (which has to be excluded, see validation above), bcl-2, and NFKB. Src seems to correlate with Caspase 3 and 9 in this experiment.

Table 1.7B: Spearman Rho Correlation calculation: In this table, the p-values of the SpearmanRho correlation calculation are presented. This analysis looks for molecules that have similar patterns of change over the timecourse of the experiment. Thus, molecules which have significant p-values are likely to change in the same general way in the experiment. This may indicate that these molecules are on the same pathway as each other.

P-value	Uninfected													
	bads112	bads153	bax	bcl2	Caspase 3	Caspase 9	IkappaBalpha	fak	jnk	lck	nfkb	p38	pyk2	src
bads112	4.96E-05	0.582143	0.934871	0.5007937	0.46180556	0.3893849	0.461805556	0.6190972	0.216171	0.299206	0.840129	0.582143	0.840129	0.500794
bads153	NA	4.96E-05	0.619097	0.1511409	0.11498016	0.2674603	0.243055556	0.5007937	0.216171	0.326835	0.036756	0.036756	0.461806	0.243056
bax	NA	NA	4.96E-05	0.1511409	0.26746032	0.196627	0.359871032	0.0217758	0.170982	0.461806	0.619097	0.934871	0.500794	0.389385
bcl2	NA	NA	NA	4.96E-05	0.70332341	0.0022321	0.840128968	0.5364087	0.619097	0.461806	0.299206	0.299206	0.793006	0.976786
Caspase 3	NA	NA	NA	NA	4.96E-05	0.4618056	0.326835317	0.359871	0.004563	0.664583	0.26746	0.11498	0.500794	0.11498
Caspase 9	NA	NA	NA	NA	NA	4.96E-05	0.881994048	0.6645833	0.427877	0.461806	0.427877	0.359871	0.840129	0.793006
IkappaBalpha	NA	NA	NA	NA	NA	NA	4.96E-05	0.6190972	0.299206	0.976786	1	1	0.036756	0.096181
fak	NA	NA	NA	NA	NA	NA	NA	4.96E-05	0.196627	0.069395	0.500794	0.664583	0.359871	0.500794
jnk	NA	NA	NA	NA	NA	NA	NA	NA	4.96E-05	0.299206	0.26746	0.11498	0.170982	0.015377
lck	NA	NA	NA	NA	NA	NA	NA	NA	NA	4.96E-05	0.096181	0.151141	0.326835	0.326835
nfkb	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	4.96E-05	0.010714	0.976786	0.326835
p38	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	4.96E-05	0.703323	0.196627
pyk2	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	4.96E-05	0.021776
src	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	4.96E-05

P-value F novicida

	bads112	bads153	bax	bcl2	Caspase 3	Caspase 9	IkappaBalpha	fak	jnk	lck	nfkb	p38	pyk2	src
bads112	4.96E-05	0.002232	0.196627	0.536409	0.0279266	0.0279266	0.243055556	0.500794	0.096181	0.083085	0.057589	0.299206	0.26746	0.389385
bads153	NA	4.96E-05	0.069395	0.299206	0.0045635	0.0022321	0.299206349	0.243056	0.083085	0.015377	0.021776	0.359871	0.083085	0.151141
bax	NA	NA	4.96E-05	0.243056	0.0072421	0.0107143	0.75203373	0.096181	0.299206	0.069395	0.461806	0.582143	0.170982	0.216171
bcl2	NA	NA	NA	4.96E-05	0.1149802	0.0830853	0.216170635	0.004563	0.096181	0.045833	0.500794	0.299206	0.021776	0.004563
Caspase 3	NA	NA	NA	NA	4.96E-05	4.96E-05	0.427876984	0.083085	0.057589	0.004563	0.11498	0.427877	0.057589	0.096181
Caspase 9	NA	NA	NA	NA	NA	4.96E-05	0.359871032	0.069395	0.036756	0.001141	0.069395	0.359871	0.027927	0.069395
IkappaBalpha	NA	NA	NA	NA	NA	NA	4.96E-05	0.461806	0.151141	0.26746	0.26746	0.007242	0.096181	0.26746
fak	NA	NA	NA	NA	NA	NA	NA	4.96E-05	0.243056	0.096181	0.752034	0.427877	0.083085	0.007242
jnk	NA	NA	NA	NA	NA	NA	NA	NA	4.96E-05	0.010714	0.151141	0.096181	0.083085	0.243056
lck	NA	NA	NA	NA	NA	NA	NA	NA	NA	4.96E-05	0.036756	0.326835	0.007242	0.045833
nfkb	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	4.96E-05	0.582143	0.057589	0.216171
p38	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	4.96E-05	0.243056	0.536409
pyk2	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	4.96E-05	0.010714
src	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	4.96E-05

Table 1.7C: Spearman Rho Correlation calculation: In this table, the Rho correlation of the SpearmanRho correlation calculation are presented. All pairs with significant P-values in the Table above have significant Rho correlations in the Table below.

Uninfected
Rho (estimate of correlation)

	bads112	bads153	bax	bcl2	Caspase 3	Caspase 9	IkappaBalpha	fak	jnk	lck	nfkb	p38	pyk2	src
bads112	1	0.227549	0.036364	0.275454	-0.3113828	0.3493976	0.311382828	-0.212125	-0.49103	-0.412129	-0.083834	-0.239525	-0.083834	-0.275454
bads153	NA	1	0.204834	0.5714286	0.61904762	0.4431217	0.476190476	0.2771285	0.5	0.409668	0.761905	0.761905	0.309524	0.47619
bax	NA	NA	1	-0.5542571	0.45786456	-0.5212217	0.373521087	0.804878	0.530159	0.29878	0.216883	0.036147	0.289178	0.361472
bcl2	NA	NA	NA	1	-0.1666667	0.9221722	0.095238095	-0.25303	-0.21429	0.301227	0.428571	0.428571	0.119048	0.02381
Caspase 3	NA	NA	NA	NA	1	-0.3113828	0.404761905	0.3855702	0.904762	0.192785	0.452381	0.619048	0.285714	0.619048
Caspase 9	NA	NA	NA	NA	NA	1	-0.071857576	-0.175761	-0.32336	0.315157	0.323359	0.38324	0.083834	-0.107786
IkappaBalpha	NA	NA	NA	NA	NA	NA	1	0.2168832	0.428571	-0.012049	0	0	0.761905	0.642857
fak	NA	NA	NA	NA	NA	NA	NA	1	0.51811	0.689024	0.277129	0.180736	0.38557	0.289178
jnk	NA	NA	NA	NA	NA	NA	NA	NA	1	0.433766	0.452381	0.619048	0.547619	0.833333
lck	NA	NA	NA	NA	NA	NA	NA	NA	NA	1	0.638601	0.554257	0.397619	0.409668
nfkb	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	1	0.857143	0.02381	0.404762
p38	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	1	0.166667	0.52381
pyk2	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	1	0.809524
src	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	1

Fnovicida
Rho (estimate of correlation)

	bads112	bads153	bax	bcl2	Caspase 3	Caspase 9	IkappaBalpha	fak	jnk	lck	nfkb	p38	pyk2	src
bads112	1	0.928571	0.52381	0.261905	0.7857143	0.7711403	0.476190476	0.285714	0.634742	0.670671	0.714286	0.428571	0.452381	0.359288
bads153	NA	1	0.690476	0.428571	0.9047619	0.9157291	0.428571429	0.47619	0.658694	0.838338	0.809524	0.380952	0.666667	0.574861
bax	NA	NA	1	0.47619	0.8809524	0.8434347	0.142857143	0.642857	0.419169	0.694623	0.309524	0.238095	0.547619	0.491027
bcl2	NA	NA	NA	1	0.6190476	0.6506496	0.5	0.904762	0.646718	0.730552	0.285714	0.428571	0.809524	0.89822
Caspase 3	NA	NA	NA	NA	1	9.88E-01	0.333333333	0.666667	0.706599	0.910196	0.619048	0.333333	0.714286	0.646718
Caspase 9	NA	NA	NA	NA	NA	1	0.373521087	0.674748	0.76365	0.951533	0.674748	0.38557	0.77114	0.6788
IkappaBalpha	NA	NA	NA	NA	NA	NA	1	0.309524	0.574861	0.443122	0.452381	0.880952	0.642857	0.443122
fak	NA	NA	NA	NA	NA	NA	NA	1	0.467074	0.634742	0.142857	0.333333	0.666667	0.874267
jnk	NA	NA	NA	NA	NA	NA	NA	NA	1	0.843373	0.562884	0.646718	0.658694	0.463855
lck	NA	NA	NA	NA	NA	NA	NA	NA	NA	1	0.754505	0.395217	0.874267	0.728916
nfkb	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	1	0.238095	0.714286	0.491027
p38	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	1	0.47619	0.263478
pyk2	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	1	0.862291
src	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	1

Conclusion: Following infection by *Francisella novicida*, the host J774A.1 cells are more activated overall than infection by Francisella LVS strain and NIH B38 strain. There are many Spearman correlations which are significant for the novicida strain infection.

Task 1.8: Analyze and compare the phosphoactivation profiles generated by *F. novicida* to vaccine (LVS) strain. Provide conclusions regarding the differences between the strains of *Francisella*.

F. LVS and F. novicida are the two more "activating" strains of Francisella in terms of host response to infection. While the B38 infection is generally very quiet, there is significant activation of signalling molecules with F. LVS infection and even more activation with F. novicida infection. This has been noted by researchers performing DNA microarray studies, that *F. novicida* is very activating to host cells (personal communication, Tularemia Meeting, March 2008).

	LVS Infected								Novicida Infected							
time in minutes	0	5	15	30	45	60	120	240	0	5	15	30	45	60	120	240
bads112	1.00	1.18	1.54	1.49	1.27	1.21	1.36	0.90	1.00	1.23	2.05	1.63	1.72	1.22	1.36	1.14
bads153	1.00	1.19	1.36	1.23	1.12	1.18	1.21	0.80	1.00	1.20	2.42	2.06	2.23	1.61	1.78	1.53
bax	1.00	1.04	1.16	1.11	1.09	1.04	1.21	1.10	1.00	1.23	1.55	1.53	1.71	1.43	1.27	1.58
bcl2	1.00	1.12	1.36	1.16	1.18	1.12	1.22	1.07	1.00	1.01	1.42	1.22	1.14	1.59	0.94	1.15
Caspase 3	1.00	1.18	1.20	1.11	1.00	0.93	1.18	0.85	1.00	1.15	2.46	2.00	2.29	1.87	1.66	1.88
Caspase 9	1.00	1.18	1.32	1.21	1.28	1.20	1.37	0.94	1.00	1.00	2.63	1.89	2.12	1.57	1.36	1.57
IkappaBalpha	1.00	1.29	1.64	1.63	1.35	1.00	1.17	1.00	1.00	1.56	1.50	1.89	1.74	1.75	1.21	0.93
fak	1.00	1.07	1.34	1.11	0.87	0.93	0.96	0.90	1.00	1.52	2.71	2.02	2.13	3.02	1.43	2.68
jnk	1.00	1.29	1.30	1.63	1.08	1.00	1.17	1.00	1.00	0.96	1.17	1.12	1.12	1.08	0.60	0.79
lck	1.00	1.14	1.34	1.20	1.28	1.15	1.30	0.87	1.00	0.67	1.52	1.38	1.22	1.20	1.00	1.15
nfkb	1.00	2.61	3.52	2.30	2.53	1.67	2.56	1.27	1.00	0.81	1.41	1.46	1.16	1.10	1.24	0.95
p38	1.00	1.31	1.55	1.38	1.02	0.68	0.95	0.73	1.00	1.11	1.03	1.15	1.79	1.18	0.90	0.83
pyk2	1.00	1.08	1.47	1.37	1.41	1.24	1.74	1.36	1.00	0.95	1.39	1.50	1.25	1.44	1.09	1.24
src	1.00	0.90	0.90	0.99	0.68	0.83	0.93	1.14	1.00	1.10	1.90	1.78	1.64	2.13	1.64	1.70

Table 1.8B: Spearman Rho Correlation calculation: In this table, the p-values of the SpearmanRho correlation calculation are presented. This analysis looks for molecules that have similar patterns of change over the timecourse of the experiment. Thus, molecules which have significant p-values are likely to change in the same general way in the experiment. This may indicate that these molecules are on the same pathway as each other.

P-value	F novicida													
	bads112	bads153	bax	bcl2	Caspase 3	Caspase 9	IkappaBalpha	fak	jnk	lck	nfkb	p38	pyk2	src
bads112	4.96E-05	0.002232	0.196627	0.536409	0.0279266	0.0279266	0.243055556	0.500794	0.096181	0.083085	0.057589	0.299206	0.26746	0.389385
bads153	NA	4.96E-05	0.069395	0.299206	0.0045635	0.0022321	0.299206349	0.243056	0.083085	0.015377	0.021776	0.359871	0.083085	0.151141
bax	NA	NA	4.96E-05	0.243056	0.0072421	0.0107143	0.75203373	0.096181	0.299206	0.069395	0.461806	0.582143	0.170982	0.216171
bcl2	NA	NA	NA	4.96E-05	0.1149802	0.0830853	0.216170635	0.004563	0.096181	0.045833	0.500794	0.299206	0.021776	0.004563
Caspase 3	NA	NA	NA	NA	4.96E-05	4.96E-05	0.427876984	0.083085	0.057589	0.004563	0.11498	0.427877	0.057589	0.096181
Caspase 9	NA	NA	NA	NA	NA	4.96E-05	0.359871032	0.069395	0.036756	0.001141	0.069395	0.359871	0.027927	0.069395
IkappaBalpha	NA	NA	NA	NA	NA	NA	4.96E-05	0.461806	0.151141	0.26746	0.26746	0.007242	0.096181	0.26746
fak	NA	NA	NA	NA	NA	NA	NA	4.96E-05	0.243056	0.096181	0.752034	0.427877	0.083085	0.007242
jnk	NA	NA	NA	NA	NA	NA	NA	NA	4.96E-05	0.010714	0.151141	0.096181	0.083085	0.243056
lck	NA	NA	NA	NA	NA	NA	NA	NA	NA	4.96E-05	0.036756	0.326835	0.007242	0.045833
nfkb	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	4.96E-05	0.582143	0.057589	0.216171
p38	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	4.96E-05	0.243056	0.536409
pyk2	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	4.96E-05	0.010714
src	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	4.96E-05

	bads112	bads153	bax	bcl2	Caspase 3	Caspase 9	IkappaBalpha	fak	jnk	lck	nfkb	p38	pyk2	src
bads112	4.96E-05	0.004563	0.069395	0.004563	0.0575893	0.0072421	0.015376984	0.196627	0.027927	0.002232	0.083085	0.11498	0.045833	0.326835
bads153	NA	4.96E-05	0.11498	0.027927	0.0107143	0.0458333	0.027926587	0.036756	0.007242	0.027927	0.036756	0.083085	0.170982	0.500794
bax	NA	NA	4.96E-05	0.027927	0.2430556	0.0575893	0.196626984	0.582143	0.151141	0.069395	0.196627	0.500794	0.002232	0.840129
bcl2	NA	NA	NA	4.96E-05	0.0575893	0.0011409	0.027926587	0.500794	0.083085	0.000397	0.021776	0.216171	0.004563	0.216171
Caspase 3	NA	NA	NA	NA	4.96E-05	0.0830853	0.045833333	0.036756	0.021776	0.057589	0.010714	0.036756	0.26746	0.619097
Caspase 9	NA	NA	NA	NA	NA	4.96E-05	0.096180556	0.619097	0.151141	0.000397	0.069395	0.389385	0.010714	0.216171
IkappaBalpha	NA	NA	NA	NA	NA	NA	4.96E-05	0.151141	0.004563	0.045833	0.036756	0.004563	0.151141	0.389385
fak	NA	NA	NA	NA	NA	NA	NA	4.96E-05	0.045833	0.461806	0.26746	0.045833	0.976786	0.703323
jnk	NA	NA	NA	NA	NA	NA	NA	NA	4.96E-05	0.11498	0.045833	0.010714	0.243056	0.752034
lck	NA	NA	NA	NA	NA	NA	NA	NA	NA	4.96E-05	0.045833	0.243056	0.015377	0.170982
nfkb	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	4.96E-05	0.11498	0.151141	0.196627
p38	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	4.96E-05	0.582143	0.793006
pyk2	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	4.96E-05	0.582143
src	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	4.96E-05

Table 1.8C: Spearman Rho Correlation calculation: In this table, the Rho correlation of the SpearmanRho correlation calculation are presented. All pairs with significant P-values in the Table above have significant Rho correlations in the Table below.

Fnovicida
Rho (estimate of correlation)

	bads112	bads153	bax	bcl2	Caspase 3	Caspase 9	IkappaBalpha	fak	jnk	lck	nfkb	p38	pyk2	src
bads112	1	0.928571	0.52381	0.261905	0.7857143	0.7711403	0.476190476	0.285714	0.634742	0.670671	0.714286	0.428571	0.452381	0.359288
bads153	NA	1	0.690476	0.428571	0.9047619	0.9157291	0.428571429	0.47619	0.658694	0.838338	0.809524	0.380952	0.666667	0.574861
bax	NA	NA	1	0.47619	0.8809524	0.8434347	0.142857143	0.642857	0.419169	0.694623	0.309524	0.238095	0.547619	0.491027
bcl2	NA	NA	NA	1	0.6190476	0.6506496	0.5	0.904762	0.646718	0.730552	0.285714	0.428571	0.809524	0.89822
Caspase 3	NA	NA	NA	NA	1	9.88E-01	0.333333333	0.666667	0.706599	0.910196	0.619048	0.333333	0.714286	0.646718
Caspase 9	NA	NA	NA	NA	NA	1	0.373521087	0.674748	0.76365	0.951533	0.674748	0.38557	0.77114	0.6788
IkappaBalpha	NA	NA	NA	NA	NA	NA	1	0.309524	0.574861	0.443122	0.452381	0.880952	0.642857	0.443122
fak	NA	NA	NA	NA	NA	NA	NA	1	0.467074	0.634742	0.142857	0.333333	0.666667	0.874267
jnk	NA	NA	NA	NA	NA	NA	NA	NA	1	0.843373	0.562884	0.646718	0.658694	0.463855
lck	NA	NA	NA	NA	NA	NA	NA	NA	NA	1	0.754505	0.395217	0.874267	0.728916
nfkb	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	1	0.238095	0.714286	0.491027
p38	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	1	0.47619	0.263478
pyk2	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	1	0.862291
src	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	1

F LVS
Rho (estimate of correlation)

	bads112	bads153	bax	bcl2	Caspase 3	Caspase 9	IkappaBalpha	fak	jnk	lck	nfkb	p38	pyk2	src
bads112	1	0.904762	0.682647	0.89822	0.6988459	0.8809524	0.829515062	0.52381	0.78072	0.928571	0.666667	0.619048	0.738095	-0.395217
bads153	NA	1	0.610789	0.778457	0.8434347	0.7380952	0.780720058	0.761905	0.87831	0.785714	0.761905	0.666667	0.547619	-0.275454
bax	NA	NA	1	0.783133	0.4787967	0.7065995	0.527696746	0.227549	0.564513	0.682647	0.526956	0.275454	0.922172	0.078313
bcl2	NA	NA	NA	1	0.6969825	0.9461247	0.773137093	0.275454	0.662689	0.970077	0.814386	0.491027	0.89822	-0.5
Caspase 3	NA	NA	NA	NA	1	0.6626987	0.728450578	0.747042	0.80253	0.698846	0.855484	0.747042	0.445815	-0.212125
Caspase 9	NA	NA	NA	NA	NA	1	0.634335047	0.214286	0.561143	0.97619	0.690476	0.357143	0.857143	-0.503003
IkappaBalpha	NA	NA	NA	NA	NA	NA	1	0.561143	0.9	0.731925	0.756323	0.902708	0.561143	-0.355889
fak	NA	NA	NA	NA	NA	NA	NA	1	0.731925	0.309524	0.452381	0.738095	0.02381	0.167668
jnk	NA	NA	NA	NA	NA	NA	NA	NA	1	0.609938	0.731925	0.853913	0.463553	-0.134992
lck	NA	NA	NA	NA	NA	NA	NA	NA	NA	1	0.738095	0.47619	0.833333	-0.538932
nfkb	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	1	0.619048	0.571429	-0.514979
p38	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	1	0.238095	-0.119763
pyk2	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	1	-0.227549
src	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	1

Multiple Tasks: Task 1.3, 1.6, 1.8:
Task 1.3: Compare the phosphoactivation profiles generated by RPMA to published results in J774A.1 cells and provide conclusions regarding the validation of RPMA technology.
Task 1.6: Analyze and compare the phosphoactivation profiles generated by the type strain B38 to vaccine (LVS) strain. Provide conclusions regarding the differences between the strains of Francisella.
Task 1.8: Analyze and compare the phosphoactivation profiles generated by *F. novicida* to vaccine (LVS) strain. Provide conclusions regarding the differences between the strains of *Francisella*.

Multiple pathways are activated during bacterial infections of macrophage. During the first few minutes of infection these pathways focus on the production proteins that will regulate pro-inflammatory cytokines, chemotaxis cytokines, apoptosis, and cytoskeleton rearrangement. The production of these proteins and events will eventually elicit a total innate immune system response. However *Francisella* bacteria have found ways to evade and/or stop the proper signaling cascade activation. *Francisella* is an intracellular pathogen that enters the cell through an unknown mechanism. Once in the cell, *Francisella* is encapsulated in a phagosome. Between 2-4 hours after the initial infection *Francisella* escapes the phagosome and proceeds to replicate in the hosts cytosol. Previous studies have demonstrated that *Francisella* inhibits the release of pro-inflammatory cytokines such as TNF-alpha, Il-1, IL-12, and IL-8 hampering the ability of the innate immune system to respond to infection. Also, there is an increase of IL-10 seen during *Francisella* infection. IL-10 is an anti-inflammatory cytokine that further decreases the innate immune system response. The lack of proper cytokine production might be caused by *Francisella*'s ability to block the TLR pathways, AKT pathways, and the apoptosis pathways, further impeding the innate immune response. Interestingly, *Francisella* does not employ the secretion systems, such as type III or type IV secretion systems, that are commonly found in pathogenic bacteria, nor does it produce toxins that could explain its ability to block cell signaling pathways.

Through the use of RPMA we will gain a better understanding of what, where, and how pathways are blocked during *Francisella* infection. This information is critical in unlocking how *Francisella* evades the immune system. For antibody selection in the RPMA, we will focus on phosphoproteins within three main pathways: the toll-like receptor (TLR) pathway, AKT pathway and apoptosis pathway. Signaling through these pathways have previously been shown to be impacted during an infection with *Francisella* and therefore a detailed molecular analysis using RPMA could provide insight into how *Francisella* is able to hamper proper cell signaling.

4) RPMA results for Individual molecules:
Caspase 9 (CC9) showed activation at 15 minutes following infection for the Francisella novicida and LVS infection. No activation in B38 infection was observed. This correlates to previous reports that state that CC9 presence is an indicator of reduced apoptosis. *Francisella* infection appears to decrease after an initial increase to cause the enhanced activation of CC9, which is an early marker for the commitment to apoptosis

Caspase 3 (CC3) showed a change seen at 15 minutes. This up regulation is then followed by an overall decrease in CC3 activation. The remainder of time points shows signaling similar to what is seen in the uninfected cells. F. novicida was the most highly activated for CC3. B38 infected cells showed an increse in C3 activity at 30 minutes. Again, like CC9, these results could indicate that the early up regulation CC3 activity induces later apoptosis during a bacterial infection.

IκBα and NF-κB displayed a different signaling profile then those of CC9 and CC3. IκBα strongly up regulated between 5-30 minutes followed by a decrease gradually tapered off. NF-κB showed a similar but slower pattern for LVS infection, which correlates with these two molecules being on the same pathway.

JNK has been identified as a possible target with which *Francisella* interferes. We see an up regulation of JNK phosphorylation between 15-30 minutes for all infeting strains of Francisella. JNK normally suppresses BCL-2 signaling. This suppression may delay the apoptotic signaling cascade.

The MAPK p38 is involved in a number of different cell signaling events including the production of pro-inflammatory cytokines and apoptosis. For Francisella LVS strain, up regulation of p38 is seen during the first 15 minutes of the infection, followed by a decrease and eventual stabilization. For the NIHB38 and *F. novicida* strain, the activation appears later, between 30-45 minutes. This could indicate that early in the *F. tularensis* LVS infection p38 is not blocked by *Francisella;* however as the infection progresses suppression of p38 occurs, resulting in a lack of cytokine production and apoptosis.

Lck is generally associated with T cell activation; however in macrophages it can be activated during IL-2 receptor stimulation and further increase IL-2 production. This in turn leads to an adaptive immune response which stimulates T cells. Lck has recently been shown to be associated with AKT pathway, although its function is not fully understood. Another explanation could be that the cell is attempting to produce IL-2 in the presence of *Francisella*. Lck is lightly activated in both F. novicida and F. LVS strains at 15 minutes post infection, and then is back to background by 2-4 hours. Thus, it appears that whatever function Lck performs is being suppressed or inactivated through its interaction with *Francisella*.
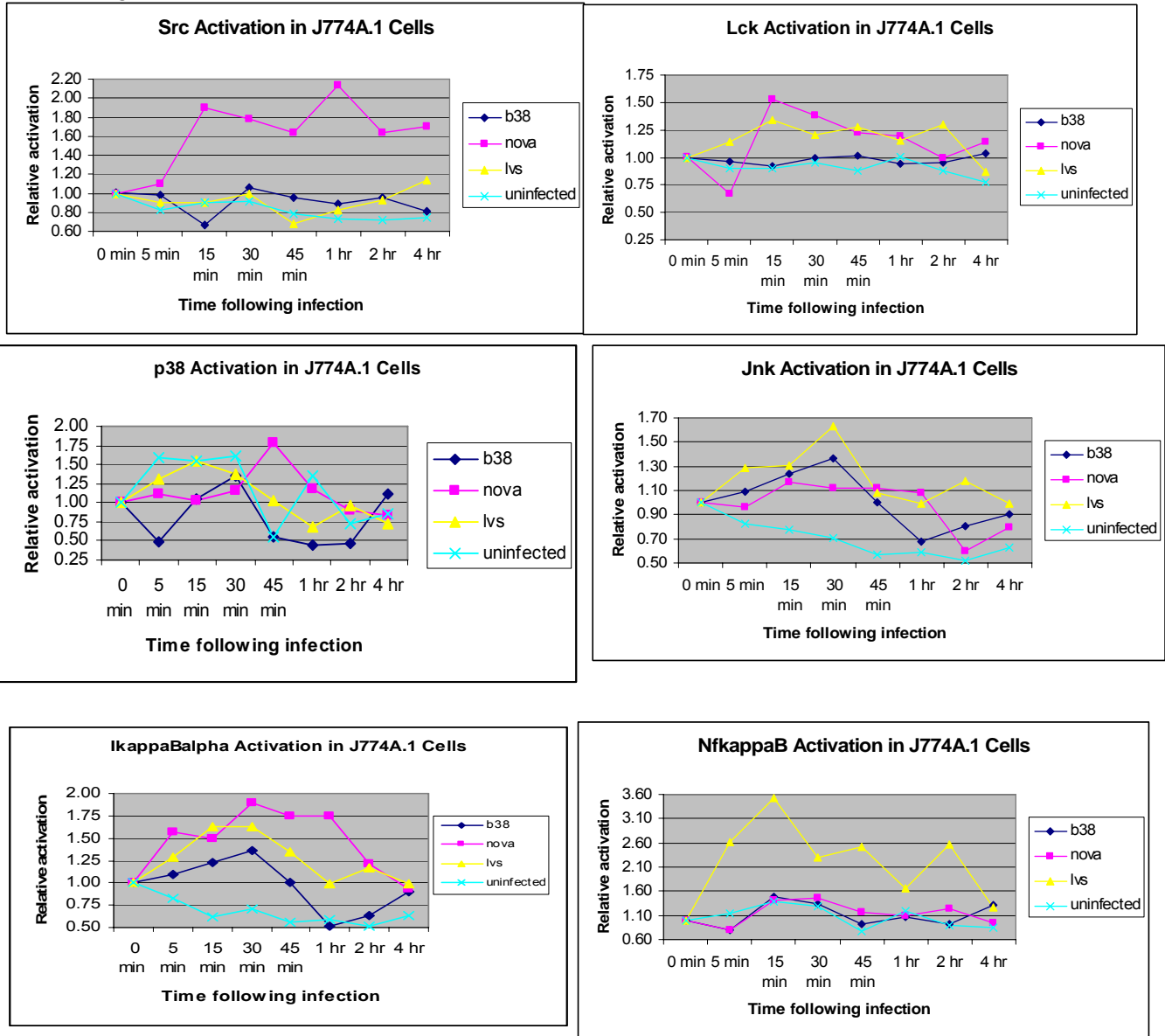
Src is involved in cell cycle, activation of Mek/Erk pathway and also in making clathrin pits. A recent study showed after *Francisella* escapes from the phagosome and replicates in the cytosol it reenters an endocytic compartment via a trafficking event. Src demonstrated an overall up regulation during *Francisella novicida* infection, but not for F. LVS and Francisella B38 infection. It is possible that the early up regulation events are due to cell cycle arrest, where as the latter events were associated with the production of this endocytic compartment.
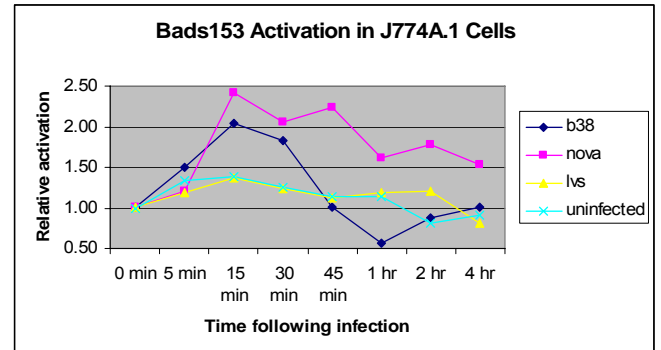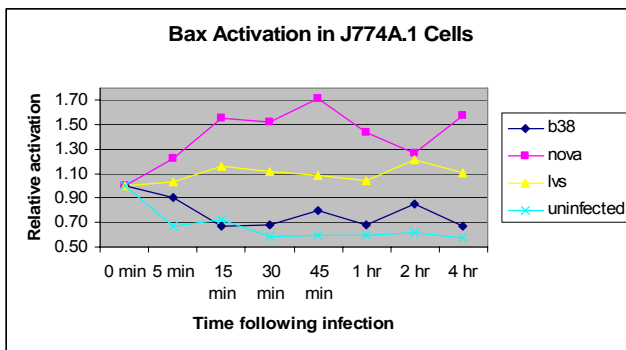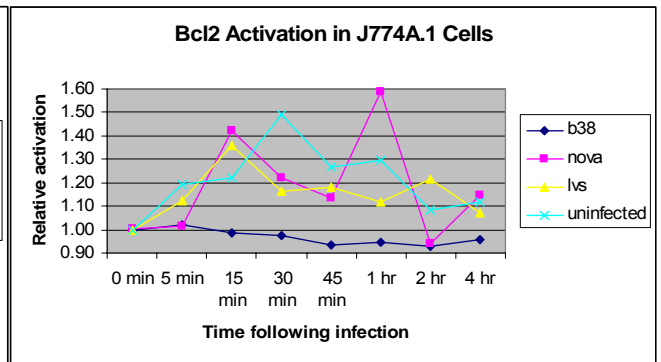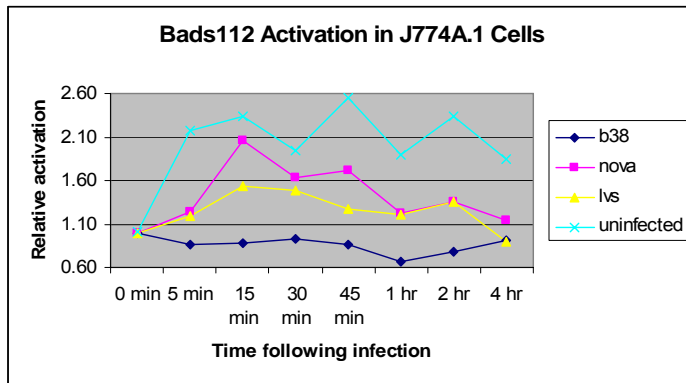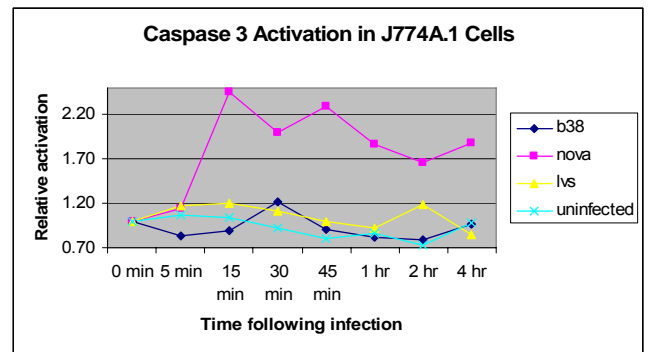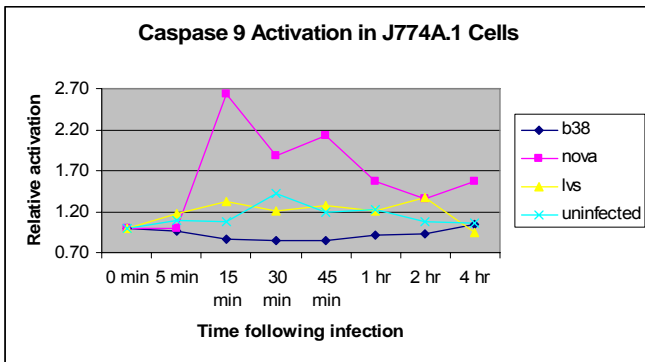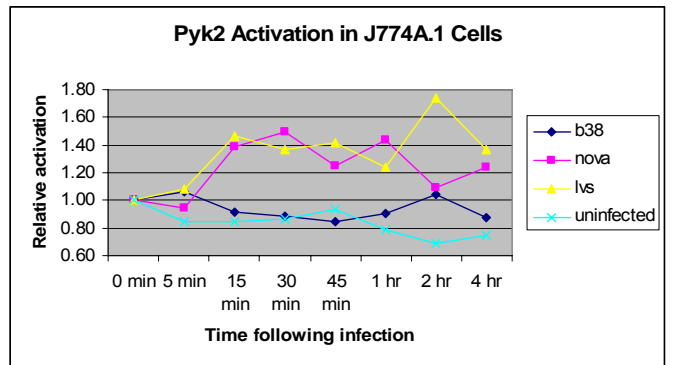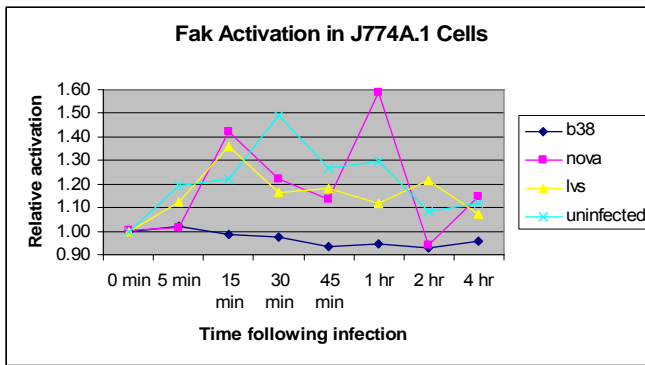
Bax is positively expressed during apoptosis, and is considered pro-apoptotic. Here Bax has a gradual up regulated trend throughout the course of the infection experiment with *F. novicida*, while remaining unchanged with *Francisella* LVS infection. This could indicate that during the later stage of *Francisella novicida* infection when it is ready to leave the cell that Bax is needed to induce an apoptotic event.

Bcl-2 negatively regulates Bax, and is considered to be anti-apoptotic. It has the effect of being anti-apoptotic. Interestingly, the Bcl-2 activation pattern appears to mimic that of Bax. Bcl-2 is bound to Bax and Bim releases Bcl-2 from Bax. It is possible that *Francisella* causes up regulation of Bcl-2 in order to suppress Bax, until apoptosis is inevitable. The B38 strain did not cause strong activation of Bcl2, while the other strains were more activating to this molecule.

Bad is bound to Bcl-2 and causes the activation of Bim. Bad can also cause the removal of Bcl-xl from Mcl-1 which in turn activates Bak. Bad has two different phosphorylation sites, Bad S112 and Bad S153, with separate biological functions. Generally, these sites are changing in parallel, as illustrated by the Spearman Correlation calculations presented throughout.

Figure 9: Correlates to Figure 9 last report. Please see graph titles for details. Relative Changes in Activation (Phosphorylation) of different molecules following *Francisella novicida* infected vs *F. tularensis* LVS infected vs *F. tularensis* NIHB38 infected vs uninfected J774A.1 macrophages.

Fak Activation in J774A.1 Cells

Pyk2 Activation in J774A.1 Cells

Caspase 9 Activation in J774A.1 Cells

Caspase 3 Activation in J774A.1 Cells

Bads112 Activation in J774A.1 Cells

Bcl2 Activation in J774A.1 Cells

Bax Activation in J774A.1 Cells

Bads153 Activation in J774A.1 Cells

Unsupervised Clustering of Heat Map Analysis: The data sets illustrated as Heat Maps represent unsupervised clustering of the infection data represented in Table S.2 in Supplemental Materials. This approach allows the computer to group the data according to the samples that are most similar in their pattern of change. This approach may reveal clustering of signalling pathways, as has been published for other experiments using RPMA (Espina et al, 2004, Liotta et al, 2003).   Heat maps are included in Tasks 1.1, 1.3 and 1.7 above, except for Uninfected, which is shown below. The Heat Maps will continue to be useful to visualize the large amount of data generated by this project. As more antibodies are tested, stronger clustering will become obvious between similarly chaning molecules.
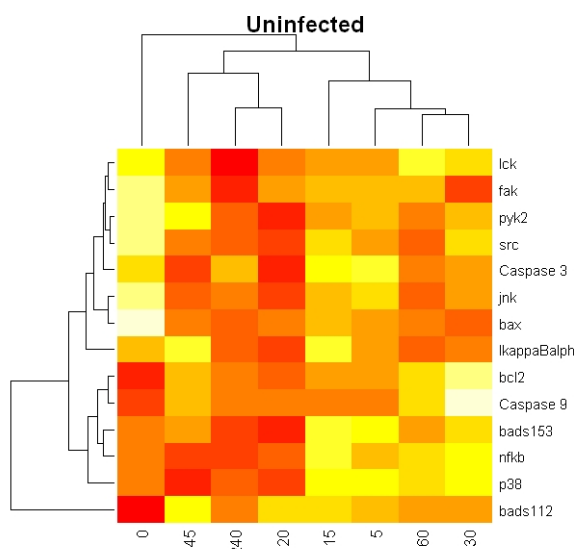
Task 2: **Screening of *Francisella* ORFs for potential VFs as a model for high-throughput screening of ORFs.**

Task 2.1: Clone and express a set of *Francisella* proteins, including *F. tularensis*, *F. novicida* and *F. tularensis* LVS genes as available (and where they are significantly different that the Ft genes). Confirm activities (if known) of expressed proteins vs. native purified proteins.

In addressing Task 2, we began the process of cloning two of the selected potential *Francisella* virulence factors that will be used to test the scientific hypothesis. The Gateway (Invitrogen Corp, Carlsbad, CA) cloning Strategy for potential virulence factors was used. A list of Virulence Factors from Francisella was developed by the Lawrence Livermore National Labs, and is attached in Supplemental Data, **Table S.1**. We have picked several proteins from this list, as well as one from the literature as model ORFs for the kinds of proteins that may be virulence factors and should be tested for their effect on host cells using RPMA.

Task 2.1.A: Cloning of AcpA, acid phosphatase: This was summarized in the Annual Report for Year 1. We will work with this construct in Year 3.

Task 2.1.B: Cloning of Tul4, the 17 kDa protein:

Tul4 (also known as lpnA, locus_tag=FTT0901) is a 17 kDa protein that is a well-known and highly reactive antigen of *Francisella* strains. It has not always been considered a virulence factor, but a recent study demonstrated that this protein plays an important role in inflammation following Francisella infection (Forestal et al 2008). It may be that this lipoprotein stimulates the TLR receptors on host cells (Thakran et al 2008). As this protein is localized on the exterior surface of the organism, it potentially interacts with host cells.

In cloning Tul4, we have used the Gateway cloning system, so that we will be able to express recombinant bacterial protein using a bacterial expression vector, as well as generate a mammalian expression vector, with which we could express this protein intracellularly inside of host cells.

The primers that were used for the Tul 4 cloning are listed below, and the cloning strategy is illustrated in Figure 2.1A.   Figure 2.1B illustrates that we were able to express the Tul4 protein from this construct, and using the epitope tags, were able to detect protien expression in bacterial colonies. The next step is to purify large quantites of this protein for use to treat host cells and perform RPMA.

Tul4 Forward Primer: 5–'GGGG **ACA AGT TTG TAC AAA AAA GCA GGC T**TC ACC *ATG* AAA AAA ATA ATC AAG CTT AGT-3'

Tul4 Reverse Primer: 5'- GGG G**AC CAC TTT GTA CAA GAA AGC TGG GT**C *GTA* AAT ATT TAT TGA ATC AGA AGC-3'

Figure 2.1A: Cloning strategy for Francisella Tul4 protein.

Figure 2.1B: Protein expression of Tul4 protein: Tul-4 was cloned into pET-DEST42 Gateway™ Vector and transformed into BL-21 competent cells. 5 colonies were selected to grow overnight and protein was extracted. 40 ug protein of each sample was subjected to SDS PAGE gel, transferred onto PVDF and blotted with anti-V5 antibody (1:5000D).

Task 2.1.C: Pilin subunit, PilE: This subtask is designed to clone and express a virulence factor (the pilE subunit from the type IV pilus) of *Francisella tularensis holarctica* LVS. The presence of type IV pilin in other organisms has been shown to be important in adhesion and colonization of an infection site. In 2004, it was discovered that LVS possessed type IV pilin like projections (Gil et al, 2004). Further analysis revealed that LVS possessed the genes necessary to produce these pilin (Gil et al, 2004). Other studies have demonstrated that some pilin subunits are directly cytotoxic to cells (Khandelwal et al, 2004). We chose to clone the *Francisella tularensis holarctica* LVS type IV pilin subunit, pilE, due to the fact that LVS is well established to make pili, and there is only one pilE subunit. In SchuS4 there are at least 5 different pilE subunits, making it difficult to identify the critical one.

Using molecular biology techniques, we will clone and express the pilE gene in a plasmid vector and express this protein subunit in E. coli. After isolating and quantifying this protein using the His tag and Talon resin, Talon™, SDS/Western Blot and immunoprecipitation will be used to characterize the pilE expression. We will then treat cultures of J774A.1 murine macrophage cells with the isolated protein, followed by examination of downstream signaling pathways using RPMA.
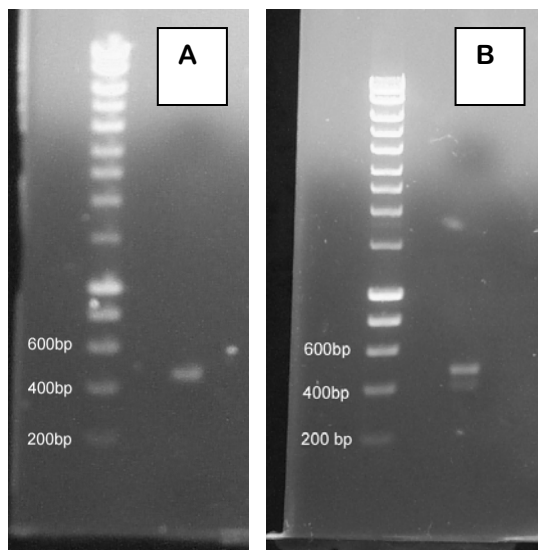
Figure 2.1.**A. Gel Electrophoresis of N-terminal tag LVS pilE subunit PCR product:** The subunit size is 408 bp. This picture indicates successful PCR of the pilE subunit as the nucleotide band falls between the 400 and 600 bp bands on the DNA ladder. This is consistent with the listed size of the subunit plus additional nucleotides (~50) to prepare this sample for cloning and expression.
B. Gel Electrophoresis of C-terminal tag LVS pilE subunit PCR product: The subunit size is 405 bp (minus UAA stop codon of N-term sample). This picture indicates successful amplification of the pilE subunit as the nucleotide band falls between the 400 and 600 bp bands on the DNA ladder. This is consistent with the listed size of the subunit plus additional nucleotides (~50) to prepare this sample for cloning and expression.

Task 2.1E: Cloning of 58 kDa protein and PLD. These proeins (58 kda protein and Phospholipase D) were also identified as potential virulence factors in Francisella. We have begun to clone these proteins also.
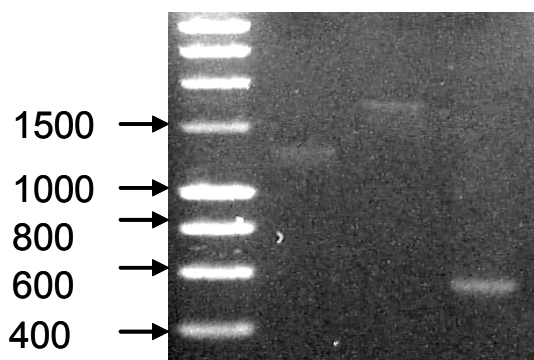

Figure 2.1E: PCR amplification of PLD, 58kDA protein and Tul-4 gene. Tul4 is discussed above. Genomic DNA was extracted from F. novicida using a genomic DNA purification kit (Promega Corporation). The primers were based on the gene sequences in F. novicida (PLD CP000439, 58kDa protein DQ230369, Tul-4 NC008301).

The primers used for the PCR cloning are shown below:
58kDa Protein Forward Primer (allowing for a C-terminal tag): 5' - GGGG ACA AGT TTG TAC AAA AAA GCA GGC TTC ACC ATG CTT TTA AAA AAC AAA TCA-3'
58kDa Protein Reverse Primer: 5'- GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC GTA TGA TTG TCT TTG TTG GAA TTT-3'
PLD Forward Primer (allowing for a C-terminal tag): 5'- GGGG ACA AGT TTG TAC AAA AAA GCA GGC TTC ACC ATG TTA GGT ACA AGA TGG AGT TGA-3'
PLD Reverse Primer: 5'- GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC ATG AGA ATT TTA TTT ACA-3'

Confirm Activity of expressed protein: Task 2.1 states in part "Confirm activities (if known) of expressed proteins vs. native purified proteins." The activity of iglC is

unknown, although it is required for intracellular growth and replication and its expression is strongly upregulated upon intracellular infection by Francisella (Telepnev et al, 2003, Ericsson et al. 1997). IglC is one of the approximately 30% of Francisella genes which have no homologies in the existing databases, and no obvious enzymatic activities or identifiable domains.

We have a hypothesis that we are testing with **iglC**. Our working hypothesis is that this protein may be either acting as a scaffold and forming complexes of proteins (bacterial and/or host) which may promote some function of intracellular bacterial growth, or that this protein receives protein phosphorylation from host-derived kinases. We will test the first hypothesis by examining which host cell proteins (if any) may associate with the iglC protein when the iglC is immunoprecipitated following incubation with host-cell lysate, or when the iglC is expressed in mammalian host cells. We will identify associated proteins by subjecting the complex to LC-MS-MS. We are testing the second hypothesis by performing in vitro phosphorylation with kinases identified as having potential sites on IglC, performing phospho-protein enrichment, and then LC-MS-MS to identify phosphorylation sites on the iglC protein. We have a unique opportunity with this technical approach to make an important contribution to the literature regarding potential binding partners and phosphorylation sites of iglC and also demonstrating the power of the technologies (RPMA and LC-MS-MS) available to us through our collaboration with the proteomics group. The coupling of infectious disease research with the tools of proteomics will lead to many such exciting discoveries.

Task 2.2. Apply *Francisella* proteins extracellularly to mouse (J774A.1 macrophages) and human (activated THP-1) cells, then generate the phosphoactivation map.

In preparation for this task, we applied LPS extracellularly to mouse J774A.1 macrophages and generated an RPMA phosphoactivation map. The results from these experiments are summarized under Task 1.2 above. We have obtained a new construct of iglC from David Waugh (Sun et al, 2007) which has increased solubility due to its construction with a maltose binding protein fusion. This fusion protein can be cleaved by an exogenous protease. We have expressed this construct and obtained iglC as confirmed by Western Blotting. We are now purifying the iglC away from the MBP fusion in preparation for application to cells.
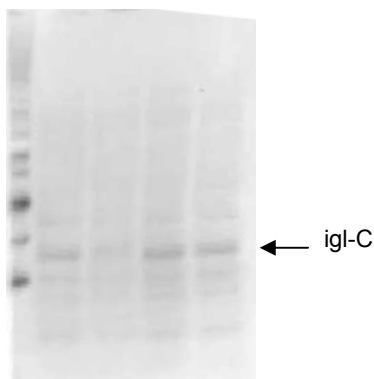
 ← igl-C

Figure 2.2. Expression of 23 kDa protein, iglC. This Western Blot demonstrates the amount of iglC we obtained using the Qiagen Mini-Xpress in vitro protein transpcription and translation system. We found this amount to be insufficient and so we obtained an expression clone from Dr. D. Waugh.

Task 2.3. Transfect plasmids expressing *Francisella* proteins into mouse (J774A.1 macrophages) and human (activated THP-1) cells, confirm intracellular expression, then generate the phosphoactivation map.

By performing RT-PCR on colonies isolated by Geneticin selection, we were able to demonstrate a high level of iglC expression in pcDNA-DEST-igl-C transfected A549 cells, prepared as described in Figure 26 of Annual Report for Year 1. This clone was not stable in long-term culture, and we eventually lost the expression of this plasmid. In the next quarter, we will be repeating the transfection and selection.
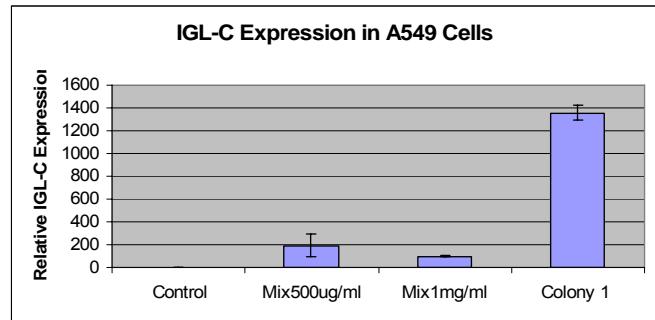
KEY RESEARCH ACCOMPLISHMENTS: Bulleted list of key research accomplishments emanating from this research.

✓ Validation study using J774A.1 cell lysate of the following antibodies for RPMA analysis:

Phospho-NFKB	Pass
Total NFKB	Pass
Total AKT (Cell Signaling 4691)	Pass
Phospho-AKT Thr 308 (Cell Signaling 2965)	Pass
Caspase 1 (Cell Signaling 2225)	Multiple Isoforms - Validated
Phospho-P42/p44 MAPK (Cell Signaling 4370)	Two Isoforms - Validated
Total p44/42 Map Kinase (Cell Signaling 4695)	Two Isoforms - Validated
Total MKK3 (Cell Signaling 9232)	Pass
Phospho-MKK3	**FAIL**
Total IRAK (Cell Signaling 4362)	Pass
pBAD-S112	Pass
Phospho-p38	Pass
Cleaved Caspase 9	Pass
Phospho-ikBa	Pass
Phospho Bcl2	Pass
Phospho-SAP/Jnk	Pass
Bax	Pass
Phospho-Paxillin	Pass
Phospho-Pyk2	**Probable FAIL**
Phospho-Src	Pass

✓
✓ RPMA of *F. tularensis LVS* infected J774A.1 macrophage cells.
✓ RPMA of *F. tularensis LVS* Lipopolysaccharide (LPS) treated J774A.1 macrophage cells.
✓ RPMA of *F. tularensis LVS* infected human activated THP-1 (macrophage) cells.
✓ RPMA of *F. tularensis B38* infected J774A.1 macrophage cells.
✓ RPMA of *F. tularensis novicida* infected J774A.1 macrophage cells.
✓ RPMA analysis of the following proteins in *F. tularensis LVS, F. novicida, F. tularensis B38* infected cells:
   o NFKB
   o IKBa
   o p38 MAPK
   o JNK
   o Caspase 9
   o Caspase 3
   o Bcl2
   o Bax
   o Bad S112
   o Bad S153
   o Fak
   o Pyk2
   o Src

- o Lck
- ✓ Comparison and correlation of RPMA results with *Francisella* infection to published results.
- ✓ Higher order analysis of data, including heat-map generation, generation of Spearman-rho correlation coefficients and p-values to identify co-changing molecules, potentially indicating pathway linkages.
- ✓ Cloning of iglC protein, a known virulence factor of *Francisella*.
  - o Bacterial expression of tagged iglC protein.
  - o Mammalian expression of tagged iglC protein is begun. Some technical issues remain.
- ✓ Cloning of the Tul4 protein, an important lipoprotein and antigen of *Francisella*.
- ✓ PCR products for cloning of additional *Francisella* potential virulence factors of Francisella: 58 kDa protein, PLD, pilE.
- ✓ Application of a bacterial product (LPS) exogenously to cells and examination of host response to isolated bacterial virulence factor.

REPORTABLE OUTCOMES: Provide a list of reportable outcomes that have resulted from this research to include: manuscripts, abstracts, presentations; patents and licenses applied for and/or issued; degrees obtained that are supported by this award; development of cell lines, tissue or serum repositories; infomatics such as databases and animal models, etc.; funding applied for based on work supported by this award; employment or research opportunities applied for and/or received based on experience/training supported by this award.

Patent application:

M.L. van Hoek, C. Bailey, S. Popov, L. Liotta, E, Petricoin. July 2007. Phosphoproteome-based approach to the identification of bacterial virulence factors. Provisional application number 60/947,231.

Poster:

van Hoek, M.L., A. B. Verhoeven, S. Han, Y.U. Taylor, L.A. Liotta, E.F. Petricoin, S.Popov and C.L. Bailey. "Phosphoproteome-based approach to the identification of bacterial virulence factors." Protein Kinases and Protein Phosphorylation Meeting, FASEB Summer Research Conference, Indian Wells, California, July 7-12, 2007.

Manuscripts in preparation:

Monique L. van Hoek, Serguei G. Popov, Emanuel F. Petricoin III, Lance A. Liotta, Charles Bailey. "Application of Reverse-phase protein Microarray to the Discovery of Novel Virulence Factors in Bacterial pathogens." *Manuscript in preparation*.

Monique L. van Hoek, SuHua Han, Annie B. Verhoeven, Yuka U. Taylor, Virginia Espina, Lance A. Liotta, Emmanuel F. Petricoin III, Serguei Popov and Charles L. Bailey. Phosphoproteome-based study of host-pathogen interactions of Francisella tularensis novicida and Live Vaccine Strain. Manuscript in preparation.

CONCLUSION: Summarize the results to include the importance and/or implications of the completed research and when necessary, recommend changes on future work to better address the problem. A "so what section" which evaluates the knowledge as a scientific or medical product shall also be included in the conclusion of the report.

In the second year of this project, we have validated a significant number of antibodies, and failed some antibodies. With this knowldege in hand for our specific system, we have processed RPMA experiments for infections of both mouse and human cells, we have infected host cells with three different strains of Francisella, and we have observed the changes for at least fourteen different molecules. Specific conclusions for these fourteen molecules are presented.

We have also applied a purified bacterial product (Francisella LPS) to host cells to observe the activation (if any) of host cell pathways. This experiment generated fascinating results, which will contribute to the field of Francisella LPS studies.

Finally, by performing a more global comparison, we observed that *Francisella novicida* is the most "activating" strain, which agrees with unpublished DNA microarray results from other groups. By using Unsupervised Clustering, Heat Maps, and Spearman Rho Correlation calculations, we are able to demonstrate important correlations between host-cell molecules which are activated following Francisella infection.

In the third year, we will focus on Task 2, while completing the manuscripts for Task 1, and performing any outstanding repeats of experiments for publication.

"So What": The utility of RPMA to map the phosphoproteome in response to bacterial infection is clearly demonstrated by the data included in this report. Furthermore, the utility of using RPMA to map activation (phosphoprotein) pathways following the application of bacterial products or virulence factors was also strongly demonstrated by the application of Francisella LPS to murine macrophages and observed the resulting changes in host-cell proteins. In terms of scientific understanding of the effect of potential virulence factors on the host cells, use of the RPMA approach will enable researchers to quickly measure changes in multiple pathways (tens of individual proteins) in a powerful, semi-automated and sensitive manner. In terms of medical advancement, it may be possible to study these pathways to find intervention points, which could have broader applicability than just the current bacteria being studied.

REFERENCES: List all references pertinent to the report using a standard journal format (i.e. format used in *Science, Military Medicine*, etc.).

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APPENDICES: Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.

Poster presented at FASEB Protein Kinase Meeting. See **Appendix 1** attachment.

SUPPORTING DATA: All figures and/or tables shall include legends and be clearly marked with figure/table numbers.

Table S1: Table of Francisella Virulence Factors from Lawrence Livermore Labs: http://greengenes.llnl.gov/kpath/bin/virulence/virulenceBrowserPublic.pl

Virulence Factor ID	Literature Name	User Designated Name	Gene Name(s)	Short Description	Long Description	Status
1277	3037072	AF055345.1	AF055345	Francisella tularensi plasmid pOM1, complete sequence		unknown
1278	AAC12935.1	AAC12935.1	AAC12935	unknown [Francisella tularensis]		unknown
1681	FTT1359c	iglA	FTT1359c iglA	unknown	required for virulence, allow survival within macrophage	virulence factor
1682	FTT1714c	iglA	FTT1714c iglA	unknown	required for virulence, allow survival within macrophage	virulence factor
1683	FTT0221	**acpA**	**FTT0221 acpA**	**phospholipase C**	**escape from phagosome**	virulence factor
1684	FTT0490c	**FTT0490c**	**FTT0490c**	**phospholipase D**	**escape from phagosome**	virulence factor
1685	FTT1249	FTT1249	FTT1249	cell entry	required for entry into macrophage	virulence factor
1686	FTT0789	rpe	FTT0789 rpe	phosphoribosyl transferase (PRTase II)	possibly involved in polysaccharide antigen synthesis	virulence factor
1687	FTT0790	FTT0790	FTT0790	glycosyltransferase	possibly involved in polysaccharide antigen synthesis	virulence factor
1688	FTT0791	galE	FTT0791 galE	UDP-glucose 4-epimerase	possibly involved in polysaccharide antigen synthesis	virulence factor
1689	FTT0792	FTT0792	FTT0792	glycosyltransferase	possibly involved in polysaccharide antigen synthesis	virulence factor
1690	FTT0793	FTT0793	FTT0793	ABC transporter, ATP-binding and membrane protein	possibly involved in polysaccharide antigen synthesis	virulence factor
1691	FTT0794	FTT0794	FTT0794	possible sugar nucleotidyltransferase	possibly involved in polysaccharide antigen synthesis	virulence factor

1692	FTT0796	FTT0796	FTT0796	LPS biosynthesis	possibly involved in polysaccharide antigen synthesis	virulence factor
1693	FTT0797	FTT0797	FTT0797	glycosyltransferase 2	possibly involved in polysaccharide antigen synthesis	virulence factor
1694	FTT0798	FTT0798	FTT0798	glycosyltransferase 2	possibly involved in polysaccharide antigen synthesis	virulence factor
1695	FTT0799	FTT0799	FTT0799	glycosyltransferase 1	possibly involved in polysaccharide antigen synthesis	virulence factor
1696	FTT0030c	fur	FTT0030c fur	transcriptional regulation	major iron response regulator	virulence factor
1697	FTT0652c	ftnA	FTT0652c ftnA	ferritin, iron storage	iron storage, iron detoxification	virulence factor
1698	FTT0651	iraB	FTT0651 iraB	possible di- and tripeptide transporter	iron acquisition	virulence factor
1699	FTT0029c	frgA	FTT0029c frgA	possible siderophore synthetase	possible iron acquisition, potentiate intracellular growth	virulence factor
1700	FTT1159c	pilN	FTT1159c pilN	export of pilus subunit, required for pilus assembly	required for pilus assembly	virulence factor
1701	FTT1156c	pilQ	FTT1156c pilQ	outer membrane pore; gated channel	required for secretion of pilin	virulence factor
1702	FTT1057c	pilF	FTT1057c pilF	pilus biogenesis	pilus biogenesis	virulence factor
1703	FTT0088	pilT	FTT0088 pilT	ATPase	pilus retraction	virulence factor
1704	FTT0230c	pilE5	FTT0230c pilE5	pilus subunits	pilin structural unit	virulence factor
1705	FTT0861c	pilE4	FTT0861c pilE4	pilus subunits	pilin structural unit	virulence factor
1706	FTT0888c	pilE3	FTT0888c pilE3	pilus subunits	pilin structural unit	virulence factor
1707	**FTT0890c**	**pilE1**	**FTT0890c pilE1**	**pilus subunits**	**pilin structural unit**	virulence factor
1708	FTT1133	pilB pilF	FTT1133 pilB pilF	ATPase	pilus extension	virulence factor
1709	FTT1134	pilC pilG	FTT1134 pilC pilG	IM protein	IM protein	virulence factor
1882	FTT0889c	pilE2	FTT0889c pilE2	pilus subunits	pilin structural unit	virulence factor
1884	FTT0683c	pilD	FTT0683c pilD	peptidase	required to produce functional pilin	virulence factor
1948	FTT1275	mglA sspA	FTT1275 mglA sspA	global transcriptional regulation, possibly through binding RNA polymerase	allow survival within macrophage and amoeba	virulence factor
1949	FTT1606	minD	FTT1606 minD	possible pump for toxic or radical ions	required for virulence, allow survival within macrophage	virulence factor
1950	FTT0795	FTT0795	FTT0795	possible Ribosomal RNA adenine dimethylase	possibly involved in polysaccharide antigen synthesis	virulence factor
1951	FTT0800	FTT0800	FTT0800	haloacid dehalogenase-like hydrolase	possibly involved in polysaccharide antigen synthesis	virulence factor

1952	FTT0801c	FTT0801c	FTT0801c	Carbohydrate/purine kinase, pfkB family	possibly involved in polysaccharide antigen synthesis	virulence factor
1953	FTT1043	FTT1043	FTT1043	immunophilin, possibly similar action to Cyclophilins (Cyps) or FKBP	immuno suppressive?	virulence factor
1954	FTT0068	sodB	FTT0068 sodB	Fe containing superoxide dismutase	iron regulated, defense against oxidative stress	virulence factor
1955	FTT1276	mglB sspB	FTT1276 mglB sspB	global transcriptional regulation, possibly through binding RNA polymerase	allow survival within macrophage and amoeba	virulence factor
1991	FTT1157c	pilP	FTT1157c pilP	pilus assembly	pilus assembly	virulence factor
2105	FTT1712c	**iglC**	**FTT1712c iglC**	**possible disruption of TLR4-mediated signal transduction in infected macrophages**	**required for intramacrophage survival of both macrophage and amoeba**	virulence factor

Table S.2: Spearman Correlation calculations, p-values and Rho values.
Spearman Rho Correlations:

P-value   Uninfected

	bads112	bads153	bax	bcl2	Caspase 3	Caspase 9	IkappaBalpha	fak	jnk	lck	nfkb	p38	pyk2	src
bads112	4.96E-05	0.582143	0.934871	0.5007937	0.46180556	0.3893849	0.461805556	0.6190972	0.216171	0.299206	0.840129	0.582143	0.840129	0.500794
bads153	NA	4.96E-05	0.619097	0.1511409	0.11498016	0.2674603	0.243055556	0.5007937	0.216171	0.326835	0.036756	0.036756	0.461806	0.243056
bax	NA	NA	4.96E-05	0.1511409	0.26746032	0.196627	0.359871032	0.0217758	0.170982	0.461806	0.619097	0.934871	0.500794	0.389385
bcl2	NA	NA	NA	4.96E-05	0.70332341	0.0022321	0.840128968	0.5364087	0.619097	0.461806	0.299206	0.299206	0.793006	0.976786
Caspase 3	NA	NA	NA	NA	4.96E-05	0.4618056	0.326835317	0.359871	0.004563	0.664583	0.26746	0.11498	0.500794	0.11498
Caspase 9	NA	NA	NA	NA	NA	4.96E-05	0.881994048	0.6645833	0.427877	0.461806	0.427877	0.359871	0.840129	0.793006
IkappaBalpha	NA	NA	NA	NA	NA	NA	4.96E-05	0.6190972	0.299206	0.976786	1	1	0.036756	0.096181
fak	NA	NA	NA	NA	NA	NA	NA	4.96E-05	0.196627	0.069395	0.500794	0.664583	0.359871	0.500794
jnk	NA	NA	NA	NA	NA	NA	NA	NA	4.96E-05	0.299206	0.26746	0.11498	0.170982	0.015377
lck	NA	NA	NA	NA	NA	NA	NA	NA	NA	4.96E-05	0.096181	0.151141	0.326835	0.326835
nfkb	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	4.96E-05	0.010714	0.976786	0.326835
p38	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	4.96E-05	0.703323	0.196627
pyk2	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	4.96E-05	0.021776
src	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	4.96E-05

P-value   F LVS

	bads112	bads153	bax	bcl2	Caspase 3	Caspase 9	IkappaBalpha	fak	jnk	lck	nfkb	p38	pyk2	src
bads112	4.96E-05	0.004563	0.069395	0.004563	0.0575893	0.0072421	0.015376984	0.196627	0.027927	0.002232	0.083085	0.11498	0.045833	0.326835
bads153	NA	4.96E-05	0.11498	0.027927	0.0107143	0.0458333	0.027926587	0.036756	0.007242	0.027927	0.036756	0.083085	0.170982	0.500794
bax	NA	NA	4.96E-05	0.027927	0.2430556	0.0575893	0.196626984	0.582143	0.151141	0.069395	0.196627	0.500794	0.002232	0.840129
bcl2	NA	NA	NA	4.96E-05	0.0575893	0.0011409	0.027926587	0.500794	0.083085	0.000397	0.021776	0.216171	0.004563	0.216171
Caspase 3	NA	NA	NA	NA	4.96E-05	0.0830853	0.045833333	0.036756	0.021776	0.057589	0.010714	0.036756	0.26746	0.619097
Caspase 9	NA	NA	NA	NA	NA	4.96E-05	0.096180556	0.619097	0.151141	0.000397	0.069395	0.389385	0.010714	0.216171
IkappaBalpha	NA	NA	NA	NA	NA	NA	4.96E-05	0.151141	0.004563	0.045833	0.036756	0.004563	0.151141	0.389385
fak	NA	NA	NA	NA	NA	NA	NA	4.96E-05	0.045833	0.461806	0.26746	0.045833	0.976786	0.703323
jnk	NA	NA	NA	NA	NA	NA	NA	NA	4.96E-05	0.11498	0.045833	0.010714	0.243056	0.752034
lck	NA	NA	NA	NA	NA	NA	NA	NA	NA	4.96E-05	0.045833	0.243056	0.015377	0.170982
nfkb	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	4.96E-05	0.11498	0.151141	0.196627
p38	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	4.96E-05	0.582143	0.793006
pyk2	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	4.96E-05	0.582143
src	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	4.96E-05

P-value   F novicida

	bads112	bads153	bax	bcl2	Caspase 3	Caspase 9	IkappaBalpha	fak	jnk	lck	nfkb	p38	pyk2	src
bads112	4.96E-05	0.002232	0.196627	0.536409	0.0279266	0.0279266	0.243055556	0.500794	0.096181	0.083085	0.057589	0.299206	0.26746	0.389385
bads153	NA	4.96E-05	0.069395	0.299206	0.0045635	0.0022321	0.299206349	0.243056	0.083085	0.015377	0.021776	0.359871	0.083085	0.151141
bax	NA	NA	4.96E-05	0.243056	0.0072421	0.0107143	0.75203373	0.096181	0.299206	0.069395	0.461806	0.582143	0.170982	0.216171
bcl2	NA	NA	NA	4.96E-05	0.1149802	0.0830853	0.216170635	0.004563	0.096181	0.045833	0.500794	0.299206	0.021776	0.004563
Caspase 3	NA	NA	NA	NA	4.96E-05	4.96E-05	0.427876984	0.083085	0.057589	0.004563	0.11498	0.427877	0.057589	0.096181
Caspase 9	NA	NA	NA	NA	NA	4.96E-05	0.359871032	0.069395	0.036756	0.001141	0.069395	0.359871	0.027927	0.069395
IkappaBalpha	NA	NA	NA	NA	NA	NA	4.96E-05	0.461806	0.151141	0.26746	0.26746	0.007242	0.096181	0.26746
fak	NA	NA	NA	NA	NA	NA	NA	4.96E-05	0.243056	0.096181	0.752034	0.427877	0.083085	0.007242
jnk	NA	NA	NA	NA	NA	NA	NA	NA	4.96E-05	0.010714	0.151141	0.096181	0.083085	0.243056
lck	NA	NA	NA	NA	NA	NA	NA	NA	NA	4.96E-05	0.036756	0.326835	0.007242	0.045833
nfkb	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	4.96E-05	0.582143	0.057589	0.216171
p38	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	4.96E-05	0.243056	0.536409
pyk2	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	4.96E-05	0.010714
src	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	4.96E-05

P-value   B38

	bads112	bads153	bax	bcl2	Caspase 3	Caspase 9	IkappaBalpha	fak	jnk	lck	nfkb	p38	pyk2	src
bads112	4.96E-05	0.299206	0.934871	0.299206	0.0011409	0.793006	0.170982143	0.196627	0.170982	0.752034	0.359871	0.045833	0.427877	0.326835
bads153	NA	4.96E-05	0.582143	0.170982	0.2674603	0.2992063	0.001140873	0.057589	0.001141	0.326835	0.389385	0.26746	0.752034	0.840129
bax	NA	NA	4.96E-05	0.619097	0.840129	0.5821429	0.881994048	0.619097	0.881994	0.664583	0.027927	0.132292	0.11498	0.069395
bcl2	NA	NA	NA	4.96E-05	0.359871	0.4618056	0.114980159	0.151141	0.11498	0.151141	0.840129	0.500794	0.326835	0.500794
Caspase 3	NA	NA	NA	NA	4.96E-05	8.82E-01	0.151140873	0.096181	0.151141	0.881994	0.299206	0.015377	0.216171	0.299206
Caspase 9	NA	NA	NA	NA	NA	4.96E-05	0.326835317	0.389385	0.326835	0.536409	0.582143	0.793006	0.326835	0.793006
IkappaBalpha	NA	NA	NA	NA	NA	NA	4.96E-05	0.015377	4.96E-05	0.389385	0.427877	0.216171	0.881994	0.389385
fak	NA	NA	NA	NA	NA	NA	NA	4.96E-05	0.015377	0.582143	0.934871	0.389385	0.752034	0.096181
jnk	NA	NA	NA	NA	NA	NA	NA	NA	4.96E-05	0.389385	0.427877	0.216171	0.881994	0.389385
lck	NA	NA	NA	NA	NA	NA	NA	NA	NA	4.96E-05	0.389385	0.703323	0.664583	0.664583
nfkb	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	4.96E-05	0.015377	0.26746	0.389385
p38	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	4.96E-05	0.132292	0.976786
pyk2	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	4.96E-05	0.582143
src	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	4.96E-05

Uninfected
Rho (estimate of correlation)

	bads112	bads153	bax	bcl2	Caspase 3	Caspase 9	IkappaBalpha	fak	jnk	lck	nfkb	p38	pyk2	src
bads112	1	0.227549	0.036364	0.275454	-0.3113828	0.3493976	0.311382828	-0.212125	-0.49103	-0.412129	-0.083834	-0.239525	-0.083834	-0.275454
bads153	NA	1	0.204834	0.5714286	0.61904762	0.4431217	0.476190476	0.2771285	0.5	0.409668	0.761905	0.761905	0.309524	0.47619
bax	NA	NA	1	-0.5542571	0.45786456	-0.5212217	0.373521087	0.804878	0.530159	0.29878	0.216883	0.036147	0.289178	0.361472
bcl2	NA	NA	NA	1	-0.1666667	0.9221722	0.095238095	-0.25303	-0.21429	0.301227	0.428571	0.428571	0.119048	0.02381
Caspase 3	NA	NA	NA	NA	1	-0.3113828	0.404761905	0.3855702	0.904762	0.192785	0.452381	0.619048	0.285714	0.619048
Caspase 9	NA	NA	NA	NA	NA	1	-0.071857576	-0.175761	-0.32336	0.315157	0.323359	0.38324	0.083834	-0.107786
IkappaBalpha	NA	NA	NA	NA	NA	NA	1	0.2168832	0.428571	-0.012049	0	0	0.761905	0.642857
fak	NA	NA	NA	NA	NA	NA	NA	1	0.51811	0.689024	0.277129	0.180736	0.38557	0.289178
jnk	NA	NA	NA	NA	NA	NA	NA	NA	1	0.433766	0.452381	0.619048	0.547619	0.833333
lck	NA	NA	NA	NA	NA	NA	NA	NA	NA	1	0.638601	0.554257	0.397619	0.409668
nfkb	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	1	0.857143	0.02381	0.404762
p38	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	1	0.166667	0.52381
pyk2	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	1	0.809524
src	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	1

F LVS
Rho (estimate of correlation)

	bads112	bads153	bax	bcl2	Caspase 3	Caspase 9	IkappaBalpha	fak	jnk	lck	nfkb	p38	pyk2	src
bads112	1	0.904762	0.682647	0.89822	0.6988459	0.8809524	0.829515062	0.52381	0.78072	0.928571	0.666667	0.619048	0.738095	-0.395217
bads153	NA	1	0.610789	0.778457	0.8434347	0.7380952	0.780720058	0.761905	0.87831	0.785714	0.761905	0.666667	0.547619	-0.275454
bax	NA	NA	1	0.783133	0.4787967	0.7065995	0.527696746	0.227549	0.564513	0.682647	0.526956	0.275454	0.922172	0.078313
bcl2	NA	NA	NA	1	0.6969825	0.9461247	0.773137093	0.275454	0.662689	0.970077	0.814386	0.491027	0.89822	-0.5
Caspase 3	NA	NA	NA	NA	1	0.6626987	0.728450578	0.747042	0.80253	0.698846	0.855484	0.747042	0.445815	-0.212125
Caspase 9	NA	NA	NA	NA	NA	1	0.634335047	0.214286	0.561143	0.97619	0.690476	0.357143	0.857143	-0.503003
IkappaBalpha	NA	NA	NA	NA	NA	NA	1	0.561143	0.9	0.731925	0.756323	0.902708	0.561143	-0.355889
fak	NA	NA	NA	NA	NA	NA	NA	1	0.731925	0.309524	0.452381	0.738095	0.02381	0.167668
jnk	NA	NA	NA	NA	NA	NA	NA	NA	1	0.609938	0.731925	0.853913	0.463553	-0.134992
lck	NA	NA	NA	NA	NA	NA	NA	NA	NA	1	0.738095	0.47619	0.833333	-0.538932
nfkb	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	1	0.619048	0.571429	-0.514979
p38	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	1	0.238095	-0.119763
pyk2	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	1	-0.227549
src	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	1

Fnovicida
Rho (estimate of correlation)

	bads112	bads153	bax	bcl2	Caspase 3	Caspase 9	IkappaBalpha	fak	jnk	lck	nfkb	p38	pyk2	src
bads112	1	0.928571	0.52381	0.261905	0.7857143	0.7711403	0.476190476	0.285714	0.634742	0.670671	0.714286	0.428571	0.452381	0.359288
bads153	NA	1	0.690476	0.428571	0.9047619	0.9157291	0.428571429	0.47619	0.658964	0.838338	0.809524	0.380952	0.666667	0.574861
bax	NA	NA	1	0.47619	0.8809524	0.8434347	0.142857143	0.642857	0.419169	0.694623	0.309524	0.238095	0.547619	0.491027
bcl2	NA	NA	NA	1	0.6190476	0.6506496	0.5	0.904762	0.646718	0.730552	0.285714	0.428571	0.809524	0.89822
Caspase 3	NA	NA	NA	NA	1	9.88E-01	0.333333333	0.666667	0.706599	0.910196	0.619048	0.333333	0.714286	0.646718
Caspase 9	NA	NA	NA	NA	NA	1	0.373521087	0.674748	0.76365	0.951533	0.674748	0.38557	0.77114	0.6788
IkappaBalpha	NA	NA	NA	NA	NA	NA	1	0.309524	0.574861	0.443122	0.452381	0.880952	0.642857	0.443122
fak	NA	NA	NA	NA	NA	NA	NA	1	0.467074	0.634742	0.142857	0.333333	0.666667	0.874267
jnk	NA	NA	NA	NA	NA	NA	NA	NA	1	0.843373	0.562884	0.646718	0.658694	0.463855
lck	NA	NA	NA	NA	NA	NA	NA	NA	NA	1	0.754505	0.395217	0.874267	0.728916
nfkb	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	1	0.238095	0.714286	0.491027
p38	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	1	0.47619	0.263478
pyk2	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	1	0.862291
src	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	1

B38

Rho (estimate of correlation)

	bads112	bads153	bax	bcl2	Caspase 3	Caspase 9	IkappaBalpha	fak	jnk	lck	nfkb	p38	pyk2	src
bads112	1	0.421687	0.030304	0.431145	0.9461247	0.1144578	0.548192771	0.526956	0.548193	0.144578	0.36747	0.728916	-0.331325	0.403614
bads153	NA	1	-0.236368	0.538932	0.4431217	-0.421687	0.957831325	0.718576	0.957831	-0.391566	0.349398	0.457831	-0.13253	0.096386
bax	NA	NA	1	0.216883	-0.0963925	0.2242465	-0.072728608	0.204834	-0.072729	0.187882	-0.787893	-0.600011	0.606072	0.690922
bcl2	NA	NA	NA	1	0.3809524	0.2994066	0.610789394	0.571429	0.610789	-0.574861	0.083834	0.275454	0.395217	0.275454
Caspase 3	NA	NA	NA	NA	1	-5.99E-02	0.574860606	0.642857	0.574861	0.059881	0.431145	0.826362	-0.503003	0.431145
Caspase 9	NA	NA	NA	NA	NA	1	-0.403614458	-0.359288	-0.403614	0.246988	-0.222892	-0.114458	0.39759	-0.114458
IkappaBalpha	NA	NA	NA	NA	NA	NA	1	0.826362	1.00E+00	-0.355422	0.319277	0.5	-0.060241	0.355422
fak	NA	NA	NA	NA	NA	NA	NA	1	0.826362	-0.227549	-0.035929	0.359288	-0.143715	0.634742
jnk	NA	NA	NA	NA	NA	NA	NA	NA	1	-0.355422	0.319277	0.5	-0.060241	0.355422
lck	NA	NA	NA	NA	NA	NA	NA	NA	NA	1	-0.349398	-0.156627	-0.192771	0.180723
nfkb	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	1	0.837349	-0.445783	-0.349398
p38	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	1	-0.596386	-0.006024
pyk2	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	1	0.240964
src	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	1

Appendix 1: Poster presented at FASEB Meeting 2007.